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Review

Cardiac trabeculation in vertebrates: Convergent evolution or evolutionary adaptations associated with heart complexity?

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ABSTRACT

One of the most important processes during early heart development is the formation of trabecular myocardium. Cardiac trabeculation is the process by which the ventricular chambers develop a complex sponge-like myocardium essential for optimal cardiac function to provide efficient oxygenation and nourishment to the developing embryo. Indeed, its importance is highlighted by the fact that defects in trabecular formation lead to embryonic lethality and congenital heart disease. In the last decades, our understanding of cardiac trabeculation in different vertebrate models has advanced significantly. However, instead of reinforcing cardiac trabeculation as a highly evolutionarily conserved process across vertebrates, these studies have identified significant differences in the way the process occurs and how it is regulated in different vertebrate species. In this review, we assembled the current knowledge on cardiac trabeculation in different vertebrate species and examined if trabecular myocardium development can be achieved through different morphogenetic processes across vertebrates or if these differences are associated with evolutionary adaptations required to develop more complex vertebrate hearts.

1. Introduction

Heart development is a dynamic and complex process that requires the precise temporal and spatial coordination of cell-cell and cell-extracellular matrix (ECM) communications to promote cardiac region-specific tissue differentiation and morphogenesis [1,2]. As soon as the primitive heart tube forms in the developing embryo, cardiomyocytes (CM), the specialised muscle cells forming the heart, start to contract resulting in the establishment of blood circulation through the embryonic circulatory system to fulfil the nourishment and oxygenation needs of the growing embryo [1,2]. Therefore, besides cellular and ECM interactions, biomechanical forces resulting from normal heart function such as wall shear-stress (WSS) from blood flow or tensile forces from cellular stretch and myocardial contraction, also play an important role in heart development [3–7].

Among the different morphogenetic processes taking place during early heart development, the formation of trabecular myocardium in the ventricular chambers is one of the most critical processes to ensure the

Abbreviations: Ang, Angiopoietin; Adamts, A Disintegrin and Metalloproteinase with Thrombospondin Motifs; AKT, Ak Strain Transforming; ATACseq, Assay for Transposase-Accessible Chromatin with Sequencing; AVC, Atrioventricular Canal; BMP, Bone Morphogenetic Protein; CCM, Cerebral Cavernous Malformations; CM, Cardiomyocyte; CRELD1, Cysteine-Rich with EGF-Like Domains 1; Cx40, Connexin 40; Dll, Delta-like Ligand; Dpf, days post fertilization zebrafish embryonic stages; E, Embryonic day mouse embryonic stages; ECM, Extracellular Matrix; Efnb2, EphrinB2; ErbB, Erythroblastic Leukemia Viral Oncogene; ERK, Extracellular Signal-regulated Kinase; FHF, First Heart Field; HA, Hyaluronic Acid; Hapln1a, Hyaluronan and Proteoglycan Link Protein 1a; Has, Hyaluronan Synthase; HBEGF, Heparin Binding EGF-like Growth Factor; HEG1, Heart of Glass 1; Hey2, Hairy and Enhancer-of-split related with YRPW motif protein 2; HH, Hamilton and Hamburger chicken embryonic stages; Hopx, Homeodomain-only Protein Homeobox; Hpf, hour post fertilization zebrafish embryonic stages; Jag, Jagged; KLF2, Krüppel-like Factor 2; LVNC, Left Ventricular Non-Compaction; Mark3, Microtubule Affinity Regulating Kinase 3; Mest, Mesoderm-Specific Transcript; Mmp2, Matrix Metalloproteinases; Myl7, Myosin Light Chain 7; NICD, Notch Intracellular Domain; NCC, Non-Compaction Cardiomyopathy; NOS3, Nitric Oxide Synthase 3; Nrg, Neuregulin; OFT, Outflow Tract; PI3K, Phosphoinositide 3-Kinase; Podx, Podocalyxin Like; RA, Retinoic Acid; RBPjκ, Recombination Signal Binding Protein for Immunoglobulin kappa J region; RNAseq, RNA sequencing; Sema, Semaphorin; SHF, Secondary Heart Field; Timp, Tissue Inhibitor of Metalloproteinases; Tnnt2, Cardiac Troponin T 2; TGFB, Transforming Growth Factor β; Vcan, Versican; VEGFA, Vascular Endothelial Growth Factor; VEGFR, Vascular Endothelial Growth Factor Receptor; WSS, Wall Shear Stress.

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proper function of the early embryonic heart. Trabeculation is the process by which the ventricular chambers develop a complex sponge-like myocardium that is essential for optimal cardiac pumping in the absence of mature cardiac valves, and for increasing the myocardial surface for oxygenation and nourishment in the absence of coronary circulation [1,2,8–11]. Indeed, defects in trabecular formation lead to embryonic lethality or congenital heart disease highlighting its importance [2,8–14]. Notably, the trabecular myocardium in mammals and birds (4 chamber heart) is also essential later in development as it contributes to the formation of the interventricular septum, the papillary muscles sustaining the atrioventricular valves, or the Purkinje fibres of the ventricular cardiac conduction system [1,2,8–11,15–17]. In addition, through the process known as trabecular compaction, the trabecular myocardium contributes to the thickening and maturation of the ventricular wall by being incorporated into the compact myocardium once the coronary circulation and cardiac valves are fully functional [2,

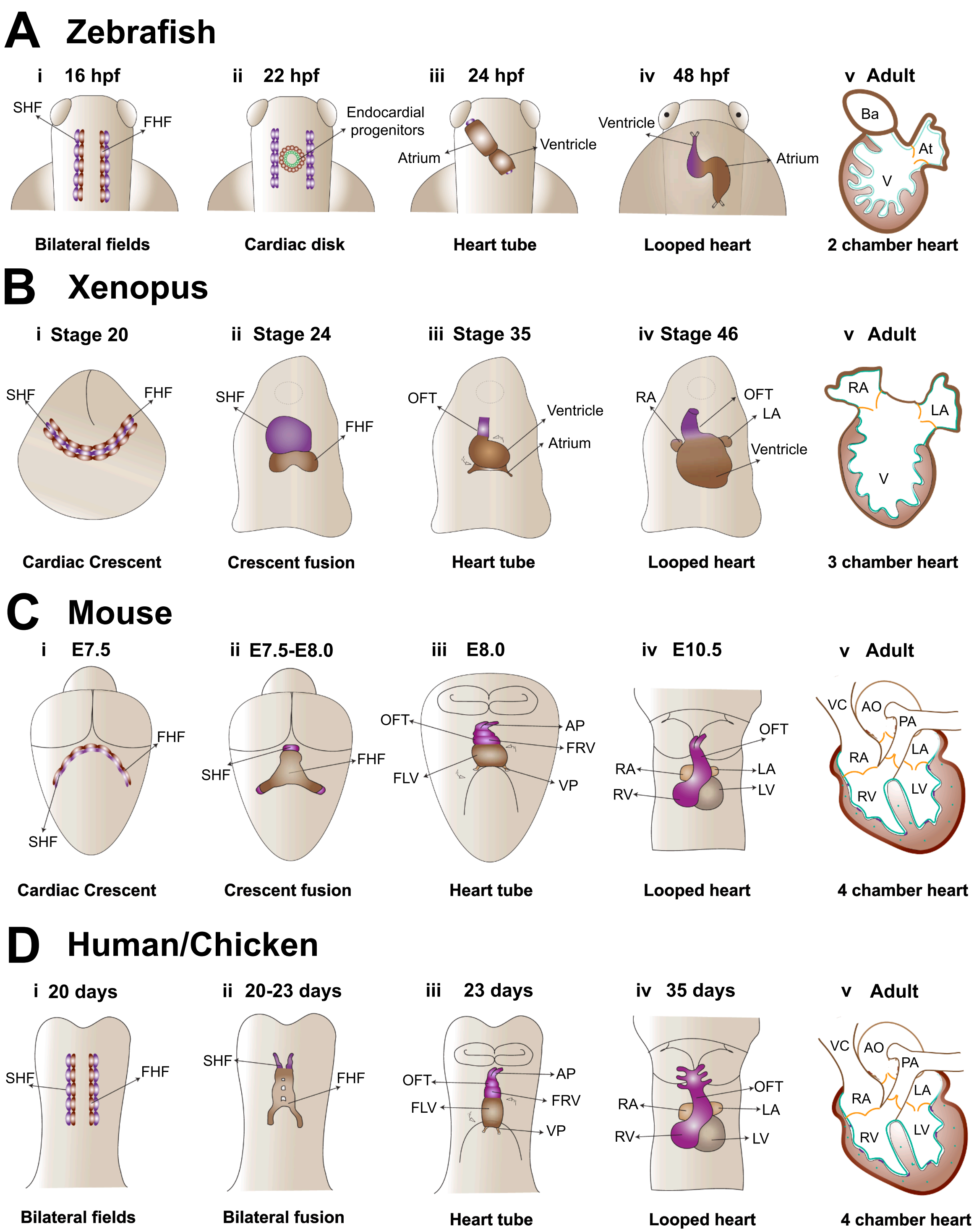

Fig. 1. Overview of early heart development in different vertebrate species. Heart development in (A) Zebrafish; (B) Xenopus; (C) Mouse and (D) Human/Chicken. First heart field (FHF); Second heart field (SHF); Bulbous arteriosus (Ba); Atrium (At); Ventricle (V); Outflow tract (OFT); Right atrium (RA); Left atrium (LA); Arterial pole (AP); Venous pole (VP); Future right ventricle (FRV); Future left ventricle (FLV); Right ventricle (RV); Left ventricle (LV); Vena cava (VC); Aorta (AO); Pulmonary artery (PA); hours post fertilization (hpf); Embryonic day (E).

8–11]. In contrast, in lower vertebrates such as the fish (2 chamber heart), amphibians and reptiles (3 chamber heart), these later contributions do not take place as the heart in these organisms does not form a septum, papillary muscles, Purkinje fibres, and does not compact, maintaining a complex trabecular network in the adult [10,18,19].

In the last decades, our understanding of the cellular, extracellular and biomechanical control of cardiac trabeculation in different vertebrate models has advanced significantly. However, instead of reinforcing cardiac trabeculation as a highly evolutionarily conserved process across vertebrates, these studies have identified significant differences in the way this process is regulated between vertebrate species. In this review, we will gather the current knowledge on cardiac trabeculation in various model organisms and analyse whether these differences are associated with our level of understanding of trabeculation in each species, if they are alternative morphogenetic processes to form trabecular myocardium, or if these differences are related to the acquisition of new developmental programs required to form more complex multichambered hearts.

1.1. *Heart development and cardiac chamber specification*

Heart development is considered to start when mesodermal progenitors located in the splanchnic mesoderm coalesce and specify becoming cardiac progenitors [1,2,20]. However, the disposition of these cardiac progenitors in the early embryo differs between vertebrate species independently of the cardiac organ complexity or class of the species [1,2,20–22]. These progenitors can coalesce forming two bilateral fields like in zebrafish (16 hours post fertilization (hpf)), chicken (Hamilton and Hamburger (HH) stage 8) or humans (20 days), or forming a crescent-shaped single field like in mice (embryonic day (E) 7.5) or xenopus (Stage 20) (Fig. 1**, Ai-Di**). Independently of their disposition, these cardiac progenitors can be divided into two main progenitor populations, the first and the secondary heart fields [1,2, 20–22]. Soon after, myocardial progenitors from the first heart field (FHF) merge in the midline of the embryo wrapping endocardial progenitors and forming the primitive heart tube [1,2,20–22]. However, the process by which FHF progenitors achieve this is also different between species. Direct FHF fusion surrounding the endocardium is the process taking place in mice and xenopus, whereas in human and chicken, the two bilateral fields form two bilateral endocardial tubes that then merge in the middle to give rise to a single cardiac tube (Fig. 1**, Aii-Diii**). In zebrafish, FHF progenitors fuse into a structure called cardiac disk that shortly afterwards elongates giving rise to the heart tube (Fig. 1**, Aii--Diii**) [1,2,20–22]. These differences suggest that during evolution, species have developed different morphogenetic processes to build the early embryonic heart from cardiac progenitors.

The primitive heart tube is formed by two concentric cellular layers, the inner endocardium and the outer myocardium, separated by a thick and highly hydrated embryonic ECM layer called cardiac jelly [1,2,20, 22,23]. The FHF cardiac progenitors forming the primitive heart tube give rise to the left or common ventricle, atrioventricular canal (AVC) and part of the atrial chambers [1,2,20,22,23]. Importantly, as soon as it forms, the primitive cardiac tube starts to pump blood to the embryonic circulatory system responding to the increasing metabolic demand of the developing embryo. Therefore, from its inception, heart development is modulated by the biomechanical forces generated by its own function [3–7].

As development proceeds, the straight cardiac tube elongates and incorporates secondary heart field (SHF) progenitors through each pole of the developing heart [1,2,20,22]. SHF progenitors contribute to the formation of the outflow tract (OFT) and part of the atrial chambers [1, 2,20,22]. In 4-chambered heart species, SHF progenitors also give rise to the right ventricle [1,2,20,22]. Simultaneously, the process of cardiac looping transforms the straight primitive cardiac tube into a looped heart with its walls disposed forming an inner and outer curvature (Fig. 1**, Aiv-Div**) [1,2,20,22]. In specific regions of the outer curvature wall, the activation of chamber specific gene regulatory networks controlling proliferation and differentiation promotes these regions to balloon out of the cardiac tube forming the ventricular and atrial chambers. In contrast, the remaining outer curvature regions and the entire inner curvature maintains features of the original myocardium forming the primitive heart tube. These regions will undergo different developmental processes leading to the formation of the cardiac valves and septa (Fig. 1**, Aiv-Div**) [1,2,22].

During chamber development, chamber CMs will differentiate into working myocardium that will hold the contractile activity of the heart [1,2,22]. However, the activation of different morphogenetic processes in the different cardiac regions results in the distinct morphological complexity of atrial and ventricular chambers. The atrial chambers are formed by a thin and smooth myocardial wall whereas the ventricular chambers develop complex myocardial structures called trabeculae (Fig. 1**, Av-Dv**). During the process of chamber specification, there are also differences among species in the way atrial and ventricular chambers form [1,2,22]. In this review, we will focus on the process of cardiac trabeculation describing the different morphogenetic processes taking place in different species to form the complex trabecular network in the ventricular chambers. In Section 1.2, we will describe the cellular behaviours and ECM dynamics taking place in the ventricular chambers during the different phases of cardiac trabeculation. In Section 1.3, we will describe the molecular regulation controlling these different phases with special focus on the endocardium-myocardium cell communication, the molecular control of ECM dynamics, and the role of biomechanical forces in the control of cardiac trabeculation.

1.2. *Cardiac trabeculation*

1.2.1. *Trabecular initiation*

Trabecular initiation is the process by which trabecular CMs first appear or specify. However, the incomplete knowledge in different model organisms makes it challenging to determine if the process of trabecular initiation is evolutionarily conserved in vertebrates.

Trabecular initiation has been studied in depth in zebrafish, and it is described to begin between 55 – 60 hpf, with trabecular CMs visible at 72 hpf [10,24,25]. In zebrafish, the ventricular chamber forms as a monolayer epithelium of CMs denominated ventricular wall from where individual CMs delaminate towards the chamber lumen to form trabecular myocardium (Fig. 2**A**) [24]. The process of delamination starts with individual CMs extending protrusions into the chamber lumen. Then, these CMs are progressively constricted by their abluminal surface and eventually exit the myocardial monolayer becoming trabecular CMs [25]. Trabecular CM delamination also requires that these delaminating CMs lose their epithelial polarity and reduce their cell-cell junctions with surrounding cells by N-Cadherin reorganization in a process similar to epithelial to mesenchymal transition (Fig. 2**A**) [26,27].

Although CM delamination in zebrafish does not involve oriented cell division of ventricular wall CMs towards the lumen [25], recent studies have identified that CM proliferation in the original ventricular CM monolayer promotes crowding of ventricular CMs. This results in a heterogeneous pattern of actomyosin tension between CMs with the ones showing higher tension levels delaminating towards the lumen to become trabecular myocardium (Figs. 2**A,** 3**A**) [28]. Even though the molecular regulation controlling trabecular initiation in zebrafish is well characterised involving cell-cell signalling intercommunication mainly through Notch and Neuregulin (Nrg) pathways [24,29–31], the absence of cardiac jelly in the ventricular chambers [31] and the presence of biomechanical forces from cardiac function [29,32], the generation of a heterogeneous CM tension pattern in the ventricular wall seems to be sufficient to promote CM delamination. This suggests that actomyosin tension patterning is the most critical factor to induce trabecular initiation (Figs. 2**A,** 3**A**) [28]. However, future studies will be required to answer several questions: (1) Is trabecular initiation a stochastic

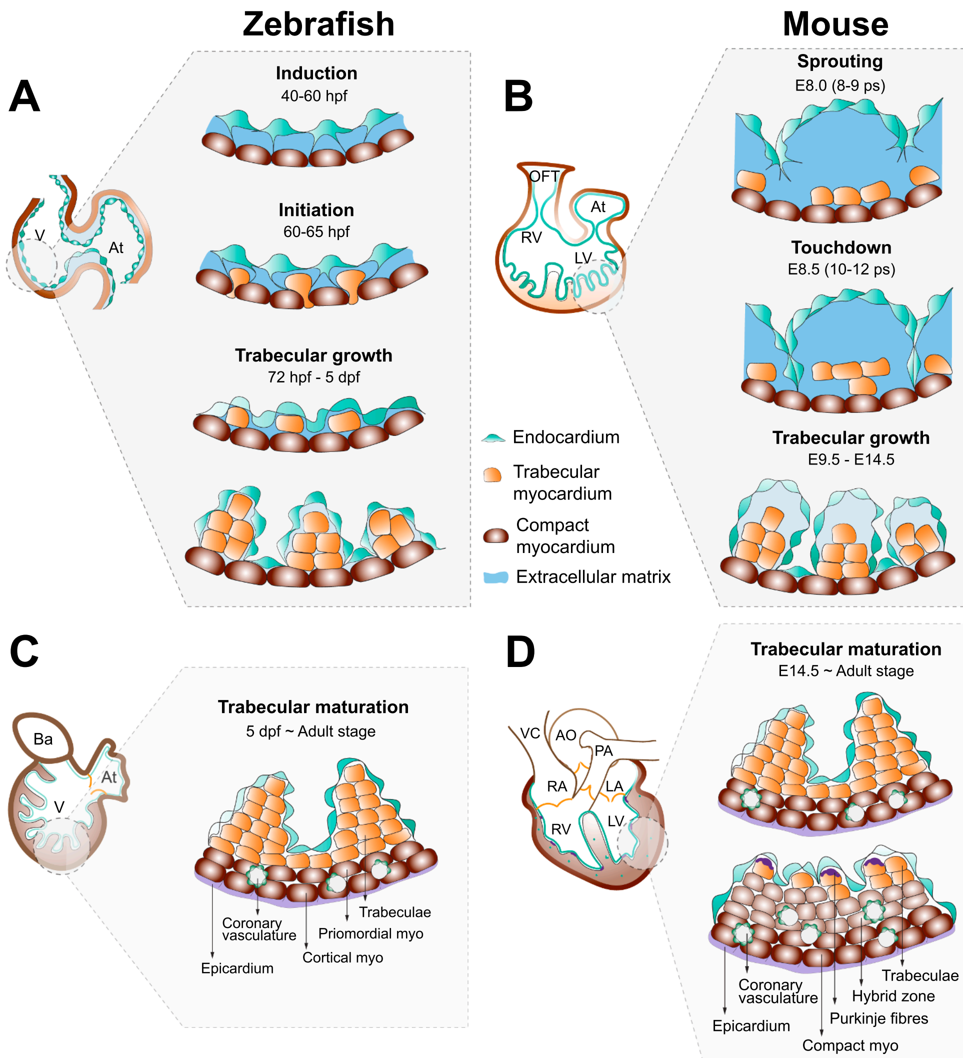

Fig. 2. Overview of cardiac trabeculation in mouse and zebrafish. Trabeculation process during early (A, B) and late (C, D) heart development in zebrafish (A, C) and mice (B, D). Bulbous arteriosus (Ba); Atrium (At); Ventricle (V); Outflow tract (OFT); Right atrium (RA); Left atrium (LA); Right ventricle (RV); Left ventricle (LV); Vena cava (VC); Aorta (AO); Pulmonary artery (PA); hours post fertilization (hpf); Embryonic day (E).

process? (2) Is the fate of delaminating CM predetermined? (3) Are the well-known cell-cell, cell-ECM and/or mechanical regulatory pathways controlling CM actomyosin tension to define which CMs become trabecular myocardium? The molecular regulation controlling trabecular initiation in zebrafish will be described in Section 1.3.

In contrast to the deep understanding in the zebrafish model, the process of trabecular initiation in other animal models is not so well understood. Indeed, until 2018, it was generally accepted that trabeculation starts after cardiac looping around E9.5 in the mouse model [1,8,9]. This stage is when the trabecular myocardium acquires its radial disposition, thus showing the sponge-like features defining this tissue (Fig. 2**B**). Also, at this stage, the expression of the defined trabecular (*Cx40, Mest, Hopx* or *BMP10*) and compact (*Hey2, N-myc*) myocardium markers is already restricted to its corresponding myocardial cell type [1,8,9]. However, a more detailed timecourse analysis showed that this restriction takes place almost 1 day early for some of these markers [14].

In 2018, a new study in mice integrating for the first time endocardial and myocardial cell behaviours and ECM dynamics taking place in the ventricular chambers from the formation of the primitive cardiac tube at E8.0, identified new critical roles for endocardium and ECM in trabeculation and pinpointed the beginning of trabeculation as early as E8.0 [14]. However, the study also highlighted that at E8.0, the myocardial epithelium forming the primitive ventricle is already multilayered and composed by a continuous outermost layer of CMs (compact layer), and clusters of luminal CMs heterogeneously distributed across the outer curvature of the ventricular wall (trabecular CMs) showing differential expression of *Has2*, *Vcan* and *VEGFa* (Fig. 2**B**) [14]. The study suggested that if the process of trabecular initiation is conserved across vertebrate species, then the process of delamination of trabecular CM from the ventricular wall monolayer described in zebrafish may be taking place before the assembly of the primitive cardiac tube in mice [14]. Importantly, this multilayered configuration can also be observed in frog, chicken and human embryos, suggesting that this may be the most common myocardial configuration across vertebrates [33–36]. Therefore, the process of trabecular initiation in other vertebrate species may occur following a different morphogenetic process by which during the formation of the primitive cardiac tube, CM progenitors form a multilayered myocardial wall as described above. Indeed, the entire myocardial wall of the primitive cardiac tube in these species is multilayered with heterogeneously distributed luminal CMs. However, only the ventricular chambers develop trabeculae in the luminal side [37]. Thus, trabecular initiation in mouse, frog, chicken and human embryos may take place by the specification of these luminal CM as trabecular myocardium induced by a ventricular-specific signalling environment. Future studies will be required to determine if trabecular CM delamination is the evolutionary conserved mechanism

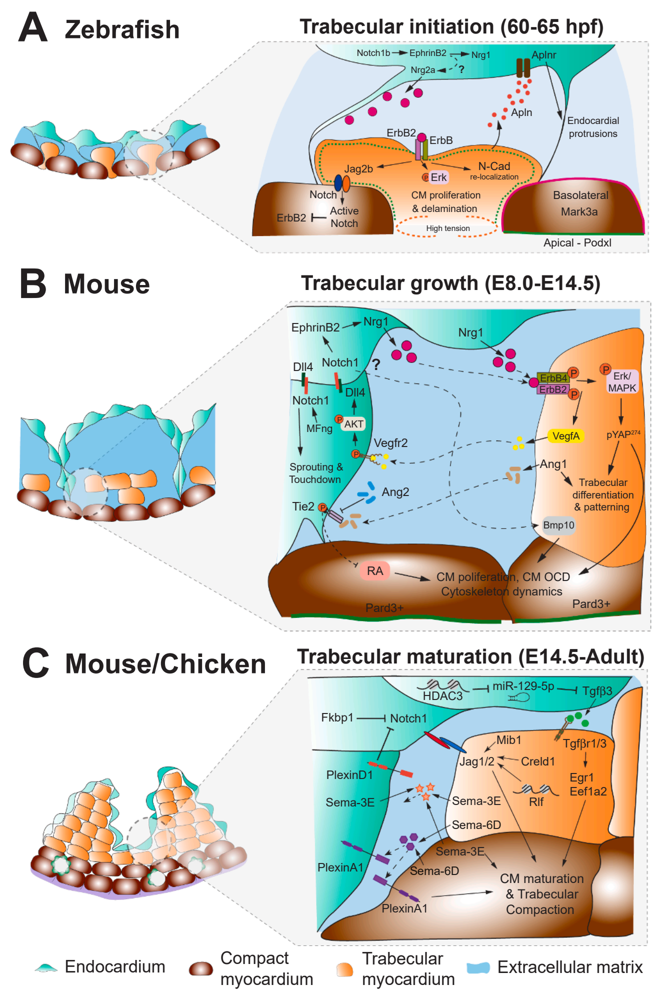

Fig. 3. Endocardium-myocardium communication during trabecular development. (A-C) Signalling pathways regulating trabecular initiation in zebrafish (A), and trabeculation growth and organization (B), and trabeculation maturation (C) in mice. Cardiomyocyte (CM), Oriented cell division (OCD).

controlling trabecular initiation in vertebrates, or instead, trabecular initiation takes place by different morphogenetic mechanisms in different vertebrate species.

The importance of the endocardium during cardiac trabeculation has been extensively documented. In fact, zebrafish *cloche* mutants, that do not form endocardium, fail to develop trabeculae [38]. Historically, studies on cardiac trabeculation have mainly focused on the myocardium with the endocardium only considered as a source of paracrine signals and mostly disregarding the ventricular ECM. However, recent studies have identified the direct involvement of endocardial behaviours and ECM dynamics in the process of trabeculation [14,31,39].

In the primitive heart tube of all vertebrates, the endocardium is separated from the myocardium by a thick cardiac jelly. Histological and electron microscopy analysis of these early hearts have identified that even though endocardium and myocardium are far apart, there are long fine subcellular protrusions connecting both tissues from these early stages [15,33,39]. However, whether these cell protrusions are anchor points to stabilise the endocardium during cardiac contraction or signalling projections/cytonemes by which endocardium-myocardium intercommunication occurs is mostly unknown. Supporting the latter, recent studies in zebrafish have described the presence of these endocardial subcellular protrusions extending towards the myocardium as early as 24 hpf (Figs. 2**A**, 3**A**) [39]. Importantly, blocking the formation of endocardial protrusions led to the inhibition of trabecular CM delamination due to the reduction of Nrg activity suggesting that these projections are indeed important for endocardium-myocardium communication during trabecular initiation in zebrafish [39]. Further studies are required to investigate if these endocardial subcellular protrusions have a similar signalling role during trabeculation in other vertebrates.

Although the primitive heart tube of all vertebrates forms with a relatively homogeneous cardiac jelly between endocardium and myocardium, soon after, as the heart loops, the distribution of cardiac jelly evolves in a cardiac region-specific manner. While a thick cardiac jelly remains in the inner curvature of the cardiac tube and the valve regions, the cardiac chambers undergo an important reduction in cardiac jelly thickness [1,2,23]. In zebrafish, the maintenance of a thick cardiac jelly characterises the atrial chamber, whereas there is a total reduction of cardiac jelly in the ventricular chamber starting at 60 hpf and ending at 96 hpf [31]. In this study, it was hypothesised that the thick atrial cardiac jelly prevents the induction of trabecular CM delamination induced by the Nrg pathway suggesting that the total cardiac jelly degradation in the ventricle is critical for trabecular initiation in zebrafish [31]. In mice, the atrium is the cardiac region undergoing total cardiac jelly degradation resulting in an atrial chamber configuration with endocardium and myocardium completely in contact with each other [23]. In contrast, the ventricular chambers undergo a finely controlled cardiac jelly reduction instructed by the endocardium generating distinct areas of thick cardiac jelly (ECM bubbles) that are critical for trabecular organization and growth. Morphological studies of cardiac development in frog, chicken and human, show a similar pattern of ECM dynamics in both atrium and ventricles suggesting that this is highly conserved in vertebrates [33–36]. Of note, total cardiac jelly degradation in ventricular chambers leads to the block of trabecular development in mice [14,40,41]. Therefore, the role of ECM in trabeculation appears to be different between fish and other vertebrates and warrants further studies to determine if these distinct ECM dynamics are simply alternative ways to form trabecular myocardium or an evolutionary adaptation needed to develop more complex hearts.

1.2.2. *Trabecular organization and growth*

In mice, recent studies have identified the endocardium as the tissue organiser controlling the location where trabeculae will form and how they are organised [14]. The study described that as early as E8.0, some endocardial cells in the outer curvature of the primitive ventricle sprout out of the endocardial layer in a process coined endocardial sprouting due to its morphological similarities with sprouting angiogenesis during vascular development [14]. These endocardial sprouts then elongate towards the myocardium becoming multicellular and forming cord-like structures without a lumen (Fig. 2**B, Sprouting**). These endocardial sprouts then tunnel through the ventricular cardiac jelly and establish cell-cell junctions exclusively with the CMs forming the outermost continuous CM layer of the ventricle (compact CMs) giving rise to the so-called endocardial touchdowns (Fig. 2**B; Touchdown**) [14]. Visualization of this process in 3D identified that these endocardial behaviours form distinct dome-like structures that isolate ECM-rich areas (ECM bubbles) in between endocardial touchdowns with the luminal CM clusters encapsulated inside these endocardial domes (Fig. 2**B; Touchdown**). As they form, the endocardial domes begin to form lumen starting from the chamber lumen end (Fig. 2**B, Touchdown**). At this point, the trabecular CM clusters are organised forming laminar structures loosely attached to the compact layer and oriented parallel to the chamber outer wall (Fig. 2**B; Touchdown**) [14]. As the endocardial touchdowns contact the compact myocardium, they extend under the laminar trabeculae further encapsulating the trabecular myocardium (Fig. 2**B; Trabecular Growth**). This promotes an important induction of trabecular growth and a change in the organization of the trabecular myocardium becoming more radially distributed towards the chamber lumen at around E9.5 during the so-called extension phase (Fig. 2**B; Trabecular Growth**) [14]. During this phase, as the trabecular myocardium grows in size and complexity, the endocardium progressively degrades the trabecular cardiac jelly from the trabecular base to its apex until it is totally degraded around E14.5 inducing trabecular growth arrest during the trabecular termination phase (Fig. 2**B; Trabecular Growth**) [14,40]. Importantly, the study demonstrated that these endocardial behaviours and ECM dynamics are critical for the organization and growth of trabecular myocardium showing that the blockage of endocardial touchdown/dome formation leads to an abnormal trabecular structure and organization, whereas the total degradation of the ventricular cardiac jelly blocks trabecular formation at early stages and promotes premature trabecular growth arrest at later stages [14,40,41]. Although similar 3D morphological analysis has not been performed in other vertebrate species, the morphological analysis performed on tissue sections suggests that similar endocardial behaviours and ECM dynamics can be observed in frog, chicken and human embryos [33–36].

As described above, the degradation of the ventricular cardiac jelly is critical for trabecular development in zebrafish and its maintenance blocks trabecular initiation [31]. However, recent studies have identified that before the total degradation of the ventricular cardiac jelly around 60 hpf, individual endocardial subcellular protrusions embrace the delaminating CMs following a similar pattern to the endocardial touchdowns described in mice (Fig. 2**A; Induction**) [39]. This implies that the endocardial behaviours taking place at the tissue level in mice may be happening in the zebrafish at the cellular level (Fig. 2**A, B**) [14, 39].

Importantly, the fact that trabecular development occurs controlled by opposite ECM dynamics in zebrafish and the rest of vertebrate species suggests species-specific requirements of ventricular cardiac jelly for cardiac trabeculation. In zebrafish, the reduction in ventricular cardiac jelly is indispensable for the endocardium-myocardium signalling critical for trabecular initiation [39]. Paradoxically, similar endocardium-myocardium communication is also required in mice and it still occurs in the presence of ventricular cardiac jelly (see below). This suggests that ventricular cardiac jelly in mouse, frog, chicken and human embryos may be required for other aspects of ventricular morphogenesis not yet identified and potentially related to the development of more complex multichambered hearts.

Regarding trabecular growth, the process of trabecular initiation in zebrafish occurs by CM delamination and not by oriented cell division of ventricular wall CMs towards the lumen [24,25]. Indeed, at 72 hpf, when trabecular initiation is induced, the CM monolayer forming the ventricular wall is more proliferative [42]. However, once trabecular CMs delaminate, the trabecular myocardium shows a higher proliferation rate than the compact layer from 96 hpf [42]. This observation implies that the main factor promoting the growth of trabecular myocardium in zebrafish is the proliferation of trabecular CMs.

In contrast, studies in mouse embryos have defined that trabecular growth mainly originates from the trabecular base, where compact myocardium CMs proliferate towards the lumen allocating new CMs to the trabecular layer [43]. From the trabecular base, the proliferation rate quickly decreases in a gradient towards the trabecular apex. Therefore, in mice, the compact myocardium has higher proliferative capacity [43]. However, these 3D studies were performed from E9.5 onwards, hence lacking the characterization of CM proliferation during the earliest phases of trabecular development. Indeed, Ki67 proliferation analysis on tissue sections at E8.5 and E9.0 identified that trabecular CMs seem to be more proliferative than at later stages suggesting that during these early stages, trabecular myocardium growth may also result from trabecular CM proliferation [14]. Importantly, recent studies in mice have provided evidence that both oriented cell division and migration contribute to cardiac trabeculation confirming the formation of trabecular CMs from compact layer CMs [44–47]. Therefore, a more detailed analysis of CM proliferation during these early stages of trabecular development is required to fully understand how trabecular growth occurs in mice. In addition, the apparent explanation for the different proliferative capacity in compact myocardium between mouse and zebrafish could be related to the fact that the compact myocardium in zebrafish remains as a monolayer until adulthood, whereas in mice the compact layer grows in thickness giving rise to a thick compact layer that then will incorporate the trabecular myocardium forming the definitive ventricular wall during later stages of heart development.

1.2.3. *Trabecular maturation*

During trabecular maturation, the trabecular myocardium evolves to contribute to the myocardium forming the adult heart. In mammals and birds, the trabecular myocardium undergoes different morphogenetic processes by which the trabecular myocardium contributes to the formation of the Purkinje fibres of the ventricular conduction system, the papillary muscles sustaining the AVC valves or the ventricular septum [22,29,48]. However, even though these cardiac components play critical functions in the adult heart, the specific morphogenetic processes and molecular regulation controlling their formation are mostly unknown.

Moreover, the trabecular myocardium undergoes compaction by which trabeculae are simplified and integrated in the compact layer to form the mature ventricular wall of the adult heart (Fig. 2**D**) [22,29]. In mice, trabecular growth is associated with the maintenance of a thick ECM bubble surrounding each trabecula [14]. As trabeculation progresses, the endocardial touchdowns will degrade progressively the trabecular cardiac jelly in a baso-apical manner until around E14.5, when the total cardiac jelly degradation triggers trabecular growth arrest in a process called trabecular termination (Fig. 2**D**) [14,22,29,40]. After trabecular termination, the complex trabecular network consolidates and undergoes trabecular compaction (Fig. 2**D**). Historically, the process was considered to occur by coalescence of the trabecular myocardium and its incorporation into the compact layer [22,29]. However, recent studies using linage tracing approaches have determined that the process of trabecular compaction takes place by active luminal growth of compact layer CMs in between trabeculae forming a hybrid zone in which a mix of compact and trabecular CMs can be found (Fig. 2**D**) [49]. However, the precise morphogenetic processes taking place during the process of compaction and their molecular regulation are mostly unknown. Importantly, abnormal termination and/or compaction processes have been associated with left ventricular non-compaction (LVNC) and non-compaction cardiomyopathy (NCC) in humans, conditions characterised by the maintenance of excessive trabeculae and a thinning of the compact layer in the adult heart [50]. This emphasizes the importance of further research to identify the specific morphogenetic processes that when defective, lead to these congenital heart disease conditions.

In the case of lower vertebrates like fish, amphibians and reptiles, the adult heart is densely trabeculated suggesting that in these species, the trabecular myocardium grows until it develops into the adult trabecular myocardium [18]. Indeed, in zebrafish, the adult trabecular myocardium maintains the expression of embryonic trabecular markers like Cx40 that become restricted to the ventricular conduction system in birds and mammals [18]. The maintenance of this immature trabecular myocardium compared to higher vertebrates may be associated with the fact that the heart of these lower vertebrate species does not form Purkinje fibres, ventricular septum, proper papillary muscles or a compacted myocardial wall, all structures derived from trabecular myocardium maturation in higher vertebrates [18,19]. During ventricular chamber maturation in zebrafish, recent studies have described the formation of a thick myocardial layer outside of the compact monolayer in adult hearts called cortical layer that clonally derives from trabecular CMs (Fig. 2**C**) [51]. Importantly, the maintenance of this fast contractile trabecular CM in lower vertebrates may suggest that the entire trabecular myocardium acts as the ventricular conduction system in these species [52].

1.3. Molecular regulation of cardiac trabeculation

1.3.1. Endocardium–myocardium communication

Although cardiac trabeculation refers mainly to the regulation of processes taking place in the myocardium, the interaction between endocardium and myocardium through paracrine and direct cell-cell communication is essential not only for the induction and specification of trabecular CM, but also for the control of endocardial behaviours and ECM dynamics controlling trabecular organization, growth and maturation.

Among all the pathways described to be involved in cardiac trabeculation, the Notch and Nrg pathways can be considered as the master regulators of the process [13,14,24,30,31,53]. The Nrg signalling pathway belongs to the epidermal growth factor family of paracrine signalling pathways in which Nrg ligands (Nrg1–4) are secreted to the ECM and activate ErbB tyrosine kinase receptors (ErbB1–4) located forming homo and heterodimers in the cellular membrane of CMs [54,55]. Ligand-receptor activation results in a cross phosphorylation of ErbB receptors leading to the activation of several intracellular signalling cascades including ERK and PI3K/AKT pathways that control cell differentiation and proliferation [54,55]. On the other hand, Notch is a cell to cell signalling pathway in which both ligands (Dll1, Dll3, Dll4, Jag1–2) and receptors (Notch1–4) are transmembrane proteins. Ligand-Receptor interaction leads to a complex process involving ligand endocytosis by the signalling cell, and a series of proteolytic cleavages in the receptor cell that result in the release of the intracellular domain of Notch receptors (NICD). NICD then translocates to the nucleus and binds to the transcription factor RbpJκ converting it from a transcriptional repressor to a transcriptional activator and triggering the expression of Notch target genes. Several reviews about Notch and Nrg pathways could be found in the literature [50,56,57]. Importantly, even though Notch and Nrg pathways are essential for cardiac trabeculation, recent studies have identified several differences in the way these pathways control the process in different vertebrate species (Fig. 3).

During trabecular initiation in zebrafish, endocardial Nrg2a is the Nrg pathway ligand involved in the molecular control of trabecular CM delamination [31]. Indeed, both *nrg2a*$^{-/-}$ and *erbb2*$^{-/-}$ mutants fail to delaminate trabecular CMs, whereas *nrg2a* overexpression promotes excessive CM delamination in the ventricle and the ectopic induction of CM delamination in the atrium [24,31]. Multiple studies have characterised the specific role of the Nrg pathway during trabecular initiation such as promoting CM proliferation critical for ventricular CM crowding, and CM apical constriction and depolarisation by redistributing cell polarity factors (Podxl and Mark3a) and N-cadherin required for trabecular CM delamination (Fig. 3**A**) [24,26,27]. A recent study has also identified that endocardial subcellular protrusions regulated by Apelin signalling are required for Nrg2a signalling to CMs and that the reduction of cardiac jelly in the ventricular chamber is critical for their function (Figs. 2**A**, 3**A**) [39].

During early heart development in zebrafish, the Notch pathway is initially active in the endocardium with *notch1b* and Notch pathway activity homogeneously observed in the ventricular and AVC/OFT endocardium from 28 hpf and becoming restricted to AVC and OFT endocardium at 72 hpf, the stage when trabecular CM can be seen in the luminal side of the ventricular chamber [29]. The study also identified that *efnb2* and *nrg1* work downstream of Notch1b and described that *notch1b* and *efnb2* morphants show trabecular defects as described in mice (see below) [29,53]. However, it is not yet known if this signalling axis also controls *nrg2a* expression (Fig. 3**A**) [31]. Interestingly, endocardial Notch1b activity dynamics in the ventricular chamber matches the formation of endocardial subcellular protrusions that embrace delaminating CMs, as well as the process of progressive cardiac jelly degradation taking place in the ventricle (Fig. 2**A**) [31,39]. However, further studies are required to determine if the Notch pathway performs similar roles in the endocardium as described in mice (see below).

Once Notch activity fades away in the ventricular endocardium at 72 hpf, it becomes active in the myocardium [30]. However, the Notch receptor responsible for this activity is still unknown. Importantly, myocardial Notch activity is restricted exclusively to the ventricular wall CMs directly adjacent to delaminating CMs and excluded from trabecular CMs (Fig. 3**A**) [30]. Notch activity then gradually fades away in the myocardial wall once trabeculation is completed [30]. This restriction in myocardial Notch activity resembles a typical Notch-dependent lateral inhibition mechanism by which Notch activity determines the fate of a cell (compact CM) whereas cells without Notch activity acquire a different fate (delaminating CM). Importantly, the activation of the Nrg pathway in ventricular wall CMs promotes the expression of *jag2a* that activates the Notch pathway in the surrounding CMs promoting a Notch-dependent blockage of *erbb2* expression [30]. This regulation results in the Nrg active cell delaminating from the ventricular CM monolayer, whereas the surrounding Notch active cells remain as compact CMs (Fig. 3**A**) [30]. Therefore, the fine cross regulation of Nrg and Notch pathway activity in compact and delaminating CMs controls trabecular initiation in zebrafish [30]. However, this Nrg/Notch regulatory process in myocardium has not been described in any other species. Although Notch2 activity has been described in the myocardium during late chamber development in mice [58], recent studies aiming to determine the potential myocardial role of Notch during early mouse heart development did not find any cardiac defects when the common Notch pathway effector RbpJκ was deleted specifically in the myocardium [59]. Importantly, in other vertebrate species, ventricular chambers form as a multilayered CM wall with CM clusters already located in the luminal side of the primitive cardiac tube [14,33–36]. Also, luminal CMs can be observed in mouse mutants for Nrg pathway components suggesting that this Nrg/Notch regulatory mechanism may be specific to promote trabecular CM delamination in fish [14,60,61].

Despite the incomplete knowledge on trabecular initiation in mice, the molecular regulation controlling trabecular myocardium differentiation, organization and growth is better understood in this model. Interestingly, the role of Notch and Nrg pathways is also essential for orchestrating trabecular growth and organization through endocardial–myocardial crosstalk [13,14,53,62]. As mentioned above, the Notch pathway seems to be only active in the endocardium with Notch1 as the main receptor driving Notch activity in mice [14,53,63,64]. However, in contrast with zebrafish in which endocardial Notch1 activity fades away around the onset of trabecular CM delamination, Notch1 activity is maintained in the ventricular endocardium throughout heart development in mice [14,29,30,53,63,64]. In fact, the dynamic endocardial activity of Notch1 has been recently demonstrated to be critical for most of the different phases of trabeculation [14]. The endocardial restriction of Notch1 activity is controlled by myocardial VEGFA activity through VEGFR2 activation in the endocardium and triggers endocardial sprouting and touchdown formation (Figs. 2**B**, 3**B**) [14]. Importantly, *Nrg1* shows a complementary endocardial expression pattern with higher expression in the luminal endocardium. Nrg1 is released from the endocardium and travels to the myocardium to activate ErbB receptors (ErbB2 and ErbB4) [55]. Analysis of the activation pattern of ErbB2 receptor identified that Nrg1 activity is enriched in trabecular CMs where it controls the expression of VEGFA (Fig. 3**B**) [14]. Furthermore, Notch1 activity has been described to control the expression of *EphrinB2,* with EphrinB2/EphB4 endocardial activity controlling *Nrg1* expression around E9.5 [53]. Therefore, the precise regulation of Notch and Nrg1 pathway activity is also essential for trabecular organization and growth in mice. However, Nrg1 and Notch pathway cross regulation appears to be stage-dependent as loss of function models of *Notch1* and *Nrg1* promote the upregulation of Nrg1 and Notch1 activities respectively until around E8.5, with *Nrg1* expression and activity becoming downregulated after this stage in *Notch1* mutants [14], phenomenon also observed in *RbpJκ* mutants [53]. Further studies will be required to better understand the intricate molecular control of Notch1 and Nrg1 pathways during the control of cardiac trabeculation (Fig. 3**B**).

Interestingly, the process of endocardial sprouting and touchdown formation resembles the endothelial cell behaviours taking place in endothelial sprouting angiogenesis during blood vessel development [14]. Furthermore, endothelial sprouting angiogenesis is also controlled by a VEGFA/Notch signalling axis, known to regulate tip/stalk cell decisions essential for this process [65]. However, whether endocardial sprouting is controlled by similar tip/stalk cell decisions has not been formally investigated. Intriguingly, besides VEGFA and Notch pathways, many other pathways involved in sprouting angiogenesis like Apelin, Tie2/Angiopoietin, BMPs, Ephrin/Eph or Semaphorin/Plexin have also been described to play a role in cardiac trabeculation, however not in endocardial sprouting [39,66–73] (Fig. 3). Indeed, mouse models with either loss of function of *Ang1*, expressing an agonist for Tie2 receptor, or a transgenic overexpression of Ang2 (antagonist of Tie2), lead to the simplification of endocardial touchdown network and trabecular disorganisation [66,67] (Fig. 3**B**). The recent description of these endocardial behaviours and the potential involvement of these signalling pathways raises the fascinating question of whether endocardial sprouting is controlled by the same molecular pathways controlling tip/stalk cell decisions during endothelial sprouting angiogenesis.

Besides the role in the control of endocardial behaviours and ECM dynamics, these pathways have been also described to be involved in the control of trabecular myocardium growth and differentiation (Fig. 3**B**). Interestingly, trabecular growth appears to be another process that differs between zebrafish and mice. In zebrafish, trabecular CMs are the main cell type promoting trabecular growth induced by Nrg2a/ErbB2 and TGFβ activity [42]. In contrast, multiple studies in mice have identified that trabecular growth takes place by oriented cell division and migration towards the cardiac lumen of compact CMs located at the base of trabeculae [44–47]. Importantly, recent studies have identified that myocardial Nrg1-dependent pErk activation orchestrates several myocardial behaviours including the establishment of apicobasal polarity, F-actin organisation, and maintaining a proper mitotic spindle position which are critical processes for oriented cell division in the control of trabecular growth in mice [47]. Among other mitogens controlling trabeculation, the Notch pathway was shown to control *Bmp10* expression in trabecular CMs promoting CM proliferation by inhibiting the activity of the negative cell-cycle regulator $p53^{kip2}$ (Fig. 3**B**) [53,69,74]. Similarly, retinoic acid (RA) has been described to control CM proliferation in response to Ang/Tie2 activity [62]. In addition, Nrg1 pathway is also known to promote CM differentiation by restricting trabecular (*Cx40, Mest, Hopx, Nppa* and others) and compact (*Hey2, Nmyc*) myocardium markers to its respective tissue [13,53].

Endocardium-myocardium communication is also crucial for trabecular maturation, remodelling and compaction at later stages (Fig. 3**C**). During these late phases of trabecular development, the Notch pathway remains as one of the most important pathways with loss of function mutants for many Notch pathway components leading to abnormal trabecular termination, maturation and compaction in mice, and associated with trabeculation related defects like LVNC and NCC in humans [50,56,57]. During trabeculation, Notch ligands and receptors are differentially distributed across endocardium and myocardium. While Dll4 ligand and Notch1 receptor are expressed in the ventricular endocardium, Jag1/2 and Notch2 are mainly present in myocardium [14,53,58,63,64]. Notch activity in the endocardium has been described as a sequential process controlled by endocardial Dll4 ligand during early trabeculation[14,53,64]. This is due to the endocardial expression of Manic Fringe (MFng), a member of the Fringe family of glycosyl-transferase involved in the post-translational modification of the Notch receptor extracellular domain. These modifications enhance Dll-Notch signalling in detriment of Jag-Notch signalling [64]. The endocardial expression of MFng from E8.5 to E11.5 promotes Dll4-Notch1 activity at these stages, whereas after E11.5, Notch1 is activated by myocardial Jag1/2 (Fig. 3**B, C**) [64]. Deletion of either one myocardial Jag1/Jag2 alone or both together, or constitutive overexpression of *Mfng* in the endocardium led to trabecular compaction defects including hypertrabeculation and reduction in compact myocardium thickness (Fig. 3**C**) [64]. In line with these findings, the mouse model with myocardial specific deletion of the E3 ubiquitin ligase Mindbomb-1 (Mib1), required for Notch signalling by promoting the endocytosis of Notch ligands, also showed trabecular compaction defects. Importantly, a single point mutation in V943F of the *Mib1* gene sequence was found in LVNC human patients (Fig. 3**C**) [75]. Additionally, the endocardial ablation of the Notch pathway regulator *Fkbp1*, which controls N1ICD protein degradation, results in ectopic activation of the Notch1 pathway also leading to trabecular compaction defects (Fig. 3**C**) [76]. Finally, *Creld1*, a relatively unknown factor associated with valve disease in humans, has been recently described to control Notch1 activity during trabecular maturation [77]. This study identified the role of Creld1 regulating *Jag1* expression in the myocardium with *Creld1* myocardial specific deletion causing decreased Notch1 activity and trabecular compaction defects similar to NCC [77]. Altogether, these studies highlight the importance of the fine regulation of Notch1 pathway activity in the control of trabecular maturation and compaction.

Semaphorin signalling has also been involved in trabecular myocardium remodelling [70,73,78–80]. Semaphorin is a complex signalling pathway in which Semaphorin ligands can be secreted or membrane associated proteins that can interact with a wide range of receptors including Plexins, Integrins, Neuropillins, Vegfrs or Erbb2 [73]. One of the earliest pieces of evidence showing the role of Semaphorin signalling in trabecular development came from the chicken model. In the chicken heart Plexin-A1 is expressed in the endocardium and compact myocardium whereas Sema6D is expressed in both compact and trabecular myocardium (Fig. 3**C**) [78,79]. Knockdown of *Sema6D* and *Plexin-A1* resulted in late ventricular chamber defects including smaller chamber size and a reduction in the trabecular network [78,79]. However, the mechanism by which Sema6D and Plexin-A1 control trabecular development is unclear. In mice, PlexinD1 is exclusively expressed in endocardial cells, whereas its ligand Sema3E is expressed in both ventricular endocardium and myocardium (Fig. 3**C**) [70]. *PlexinD1* knock-out mice display severe heart defects including abnormal trabecular development [80]. In addition, endothelial specific deletion of *PlexinD1* showed hyper-trabeculation and non-compaction defects [70]. The study identified an upregulation of Notch1 and Nrg1 signalling pathways in *PlexinD1* mutants. Intriguingly, pharmacological inhibition of Notch pathway activity partially rescued *PlexinD1* mutant cardiac defects suggesting that Semaphorin/Plexin-D1 signalling may be involved in the control of Notch1 pathway activity during trabecular maturation and compaction (Fig. 3**C**) [70].

Beside signalling pathways, recent studies have identified a link between epigenetic modulators and trabeculation [71,81]. Loss of function of epigenetic modifier *Rlf* in mice caused the reduction of myocardial Jag1 ligand expression leading to the reduction of Notch1 activity in the endocardium and cardiac defects similar to LVNC (Fig. 3**C**) [81]. Similarly, endothelial specific deletion of *Hdac3* identified the transcriptional regulation of miR-129–5p by HDAC3 activity [71]. MiR-129–5p is known to control Tgfβ3, a critical endocardial factor controlling CM proliferation through Tgfbr1/3 in the myocardium [82], preventing its secretion and leading to trabecular maturation defects (Fig. 3**C**) [71]. Finally, recent gene loss of function studies have identified Sox7 as a new factor involved in trabecular maturation and compaction [83]. However further studies are required to determine its position in the gene regulatory network controlling cardiac trabeculation.

1.3.2. *Molecular control of ventricular ECM dynamics*

The cardiac ECM provides structural, biochemical, and physiological support for the cell types forming the cardiac chambers. Previously considered as a purely structural support for the cells, the ECM is now recognised as an important hub for biomechanical signal transduction regulating multiple cell behaviours including adhesion, migration, proliferation, differentiation and death. The cardiac ECM is formed by a complex network of fibrillary proteins (collagens), elastic fibres (elastins), glycoproteins (fibronectin, proteoglycans, laminins) comprising the "core matrisome" and a large number of ECM-associated proteins and glycosaminoglycans [84–86]. Cardiac cells interact with ECM components by ECM adhesion receptors including integrins, syndecans and discoidin domain receptors among others, providing the coupling between ECM and the intracellular cytoskeleton [87].

The role of ECM dynamics during the process of cardiac trabeculation has been recently described [14,31]. Before, most studies on the cardiac jelly were mainly focused on the valve regions ignoring the potential role of the ventricular cardiac jelly or assuming its total absence in the ventricular chambers. Indeed, a recent study identified that the latter is the case in zebrafish, which requires ventricular cardiac jelly degradation for proper ventricular development [31]. The study also identified that the absence of ventricular cardiac jelly is critical to provide the proximity between endocardium and myocardium needed for Nrg-dependent induction of CM delamination during trabecular initiation [31,39]. Furthermore, the maintenance of a thick cardiac jelly prevents trabeculation in the atrial chamber and failure to degrade the ventricular cardiac jelly leads to trabecular initiation block highlighting the critical role of ECM dynamics in the process. Therefore, cardiac trabeculation in zebrafish takes place with a total absence of cardiac jelly between endocardium and myocardium. However, although the composition of the cardiac ECM in zebrafish is described to be similar to other vertebrates, the molecular regulation controlling ventricular ECM synthesis and degradation in zebrafish is mostly unknown [88]. In this regard, a study has correlated the asymmetric localization of the ECM crosslink protein Hapln1a (Hyaluronan and proteoglycan link protein 1a) as the main factor regulating the differential cardiac jelly distribution between atrium and ventricle [89]. However, further studies are required to identify other factors involved in the control of ECM dynamics in zebrafish.

In mice, the molecular control of ECM dynamics during trabeculation

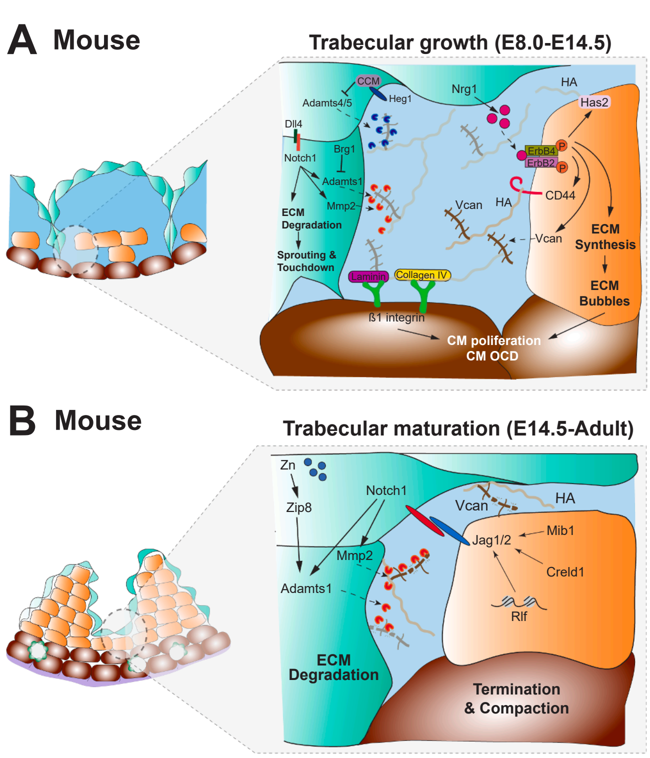

Fig. 4. Molecular control of ventricular ECM dynamics (A-B) Signalling pathways regulating ECM dynamics during trabecular growth (A) and trabecular maturation (B) in mice. Cardiomyocyte (CM), Oriented cell division (OCD).

is better understood and it is mainly regulated by the complementary roles of the Notch and Nrg pathways (Fig. 4) [14]. During trabeculation, the proper balance between ECM degradation and synthesis is crucial not only for endocardial sprouting and touchdown formation, but also to establish the location where ECM bubbles and hence, trabeculae will form in the ventricular chamber. During the process of endocardial sprouting and touchdown formation, the restriction of Notch1 activity in the endocardial cells undergoing sprouting activates the expression of ECM degradation proteins that facilitates the ingression and migration of endocardial sprouts through the ventricular cardiac jelly resulting in the formation of endocardial touchdowns [14]. During trabeculation, the endocardium is the main tissue promoting ECM degradation by activating the expression of matrix metalloproteinases from the ADAMTS and MMP families. In particular, Notch1 activity has been described to control *Mmp2* expression and directly regulates the transcription of *Adamts1* (Fig. 4**A**) [14]. In contrast, the Nrg1 pathway controls ECM synthesis in the myocardium by regulating the expression of genes involved in the formation of ECM like *Has2* or *CD44,* involved in the hyaluronic acid (HA) synthesis and signalling, or *Vcan,* encoding for the proteoglycan Versican that also interact with HA (Fig. 4**A**) [14]. Of note, these ECM synthesis markers are enriched in the luminal CM at E8.0, but this restriction is independent of Nrg1 activity at these early stages [14]. However, Nrg1 activity is critical for the maintenance of their expression in the trabecular apex from E8.5 onwards [14]. Importantly, endocardial *Notch1* loss of function promotes a reduction of ECM degradation inhibiting endocardial sprouting and touchdown formation and the accumulation of a thicker cardiac jelly in the ventricular chambers. However, this excessive cardiac jelly is not only due to defective ECM degradation but also promoted by an excessive ECM synthesis due to the upregulation of Nrg1 activity in the myocardium of these mutants. Similarly, *Nrg1* loss of function mutants show a total degradation of the ventricular cardiac jelly resulting in the block of trabecular development. Again, this phenotype is not only associated with the Nrg1-dependent reduction of ECM synthesis, but also due to the excessive ECM degradation caused by ectopic activation of Notch1 in the endocardium. Therefore, the fine regulation of the complementary control of ECM synthesis and degradation by Nrg1 and Notch1 pathways respectively is crucial for endocardial sprouting and touchdown formation, but also for trabecular organization and patterning in mice [14]. Furthermore, this complementary role of the Notch and Nrg1 pathways is also critical in the control of trabecular growth and termination. The progressive restriction of Nrg1-dependent ECM synthesis to the trabecular myocardium apex together with the progressive expansion of Notch1-dependent ECM degradation from the endocardium of the trabecular base to the apex leads to the restriction of ECM bubbles to the trabecular apex until around E14.5 when the total degradation of the ventricular cardiac jelly leads to trabecular growth arrest (trabecular termination) [14]. Indeed, a pick of *Adamts1* expression around E12.5 promotes the total degradation of ECM bubbles and trabecular termination [40].

As mentioned above, while trabecular development requires the total ventricular cardiac jelly degradation in zebrafish, the maintenance of ECM bubbles in mice is crucial to maintain trabecular growth. In fact, loss of function mouse mutants for ECM synthesis genes including *Has2* [90] or *Vcan* [91] perfectly phenocopy the *Nrg1* mutant phenotype, showing ventricular chambers completely depleted from cardiac jelly and trabecular myocardium. Similarly, mutants causing the ectopic expression of ECM degradation genes such as *Adamts1, Adamts4* and *Adamts5*, lead to the total degradation of the ventricular cardiac jelly preventing trabecular development when affecting the early phases of trabeculation, or a premature trabecular growth arrest when affecting later stages [14,40,41]. In addition, besides the Notch1 pathway, other regulatory mechanisms have been associated with the regulation of these ECM degradation genes. Endocardial *Brg1* has been described to control trabecular development by negatively regulating *Adamts1* expression, with loss of *Brg1* resulting in *Adamts1* overexpression which degrades the ventricular cardiac jelly causing premature trabecular termination (Fig. 4**A**) [40]. Also, the CCM (cerebral cavernous malformation) pathway has been described to block *Adamts4* and *5* expression during cardiac trabeculation (Fig. 4**A**) [41]. This study observed that the endocardial deletion of CCM pathway components in mice promoted the ectopic expression of *Adamts4* and *5* resulting in the total loss of ventricular cardiac jelly and premature trabecular termination [41]. This regulatory mechanism has also been demonstrated in zebrafish [92]. In addition, studies have also described similar cardiac defects associated with the loss of function of integrins, cellular receptors attaching cells to the ECM [93,94]. Using different myocardial specific loss of function models for *Itgb1* (β1 integrin), these studies described hypotrabeculation phenotypes as early as E9.5 [94] or at E12.5 [93] associated with abnormal proliferation (Fig. 4**A**). Interestingly, the phenotype of the more severe model resembles the *Nrg1* mutant phenotype with complete blockage of trabeculation and a complete reduction of the ventricular cardiac jelly [94]. The study identified that the lack of β1 integrin interaction with its ECM ligands laminin 411 and collagen IV caused defects in the specification of trabecular and compact CMs leading to abnormal oriented cell division critical for trabecular development (Fig. 4**A**) [94].

Importantly, studies on loss of function mutants for ECM degradation genes including *Adamts1*, the metalloprotease cofactor *Fibulin 1*, or genes known to control the expression of ECM degradation genes like Notch1 pathway genes or the novel zinc transporter *Slc39a8* (Zip8), have identified the critical importance of the process of trabecular termination for the subsequent process of trabecular compaction and ventricular chamber maturation (Fig. 4**B**) [14,40,64,75–77,81,95,96]. Indeed, all these mutants cause the downregulation of different ECM degradation genes required for the total degradation of the ventricular cardiac jelly during trabecular termination leading to the maintenance of trabecular growth and abnormal trabecular compaction. This results in cardiac defects like LVNC and NCC in humans, characterised by hypertrabeculation and a thinning of the compact myocardium [14,40,64,75–77,81,95,96]. Altogether, these studies highlight the importance of proper ECM dynamics for the different phases of trabeculation and its implication for congenital heart disease.

1.3.3. Biomechanical regulation of cardiac trabeculation

Besides the signalling regulation between cells or between cells and ECM, heart development is also modulated by its own function [3–7]. Indeed, cardiac contraction produces different mechanical forces that have been identified as critical for multiple processes during heart development including the establishment of the first coordinated heartbeat, or the processes of cardiac chamber morphogenesis, valve development and cardiac trabeculation [3–7]. These mechanical forces include endocardial wall shear stress (WSS) produced by the blood flow, and the tension stress associated with cellular stretching due to fluid pressure or CM contraction that promote membrane deformations in the cells forming the different cardiac tissues [3–7]. These forces are sensed through the process of mechanotransduction by which cells sense mechanical changes in their surroundings and transform these stimuli into intracellular chemical signals that activate or modulate biomechanically regulated cellular processes [3–7]. Cellular mechanosensors are usually membrane proteins able to change their structure in response to cell membrane deformations and promote the activation of intracellular signalling cascades. The main mechanosensors described are stretch-activated cation channels able to open a mechanosensitive pore creating a cation influx inside the cell, or cell-cell/cell-ECM adhesion proteins that sense cellular tension or ECM stiffness triggering cellular responses [3–7]. Most of these mechanosensitive proteins can also be associated with specialised membrane structures like caveolae or primary cilia, known to act as subcellular mechanosensing structures, or with the actomyosin network, involved in cellular contraction and cell shape changes [3–7].

Most of our understanding of the role of biomechanical forces during heart development has been generated in animal models with external development like zebrafish, frogs and chicken. This is mostly due to their accessibility for manipulation and their optimal conditions to perform live imaging studies to measure these forces and identify the cardiac processes affected by them. In particular, its complex genetic tools, its optimal live imaging capabilities and its capacity to survive without a beating heart until 7dpf by oxygen diffusion, have placed the zebrafish as the model of choice for this type of studies [97]. In zebrafish, both contractile forces and blood flow contribute to the regulation of chamber morphogenesis by controlling CM size and shape, as well as endocardial proliferation [98–100]. Importantly, studies in zebrafish have identified that fluid forces play an essential role in cardiac trabeculation. In the zebrafish ventricular chambers, it has been estimated that the endocardium surrounding the trabecular apex is exposed to higher and more unidirectional WSS, whereas the endocardium located at the trabecular base is exposed to lower and more oscillatory WSS (Fig. 5) [101,102]. Indeed, the lower blood flow WSS induced by the reduction of blood cells in *gata1* morphants and mutants or reduced atrial contraction in *weak atrium* mutants among other models, results in a total block of trabecular CM delamination suggesting that appropriate WSS and CM contraction is essential to induce trabecular initiation in zebrafish (Fig. 5) [25,103,104]. Also, intracellular forces, like the ones resulting from the contraction of the actomyosin network, have been recently described as a critical factor controlling trabecular CM delamination in zebrafish (Fig. 5) [28].

Several signalling pathways critical for trabecular initiation have been identified to be controlled by biomechanical forces in zebrafish. CM contractility is essential for the expression of Nrg pathway components including *erbb2* and *nrg2a* suggesting that the lack of trabeculation in the absence of cardiac contraction may be due to the loss of Nrg signalling [31,103]. However, recent studies have determined that the process of CM apical constriction and depolarization required for the delamination of trabecular CM is also controlled by blood flow and cardiac contraction independently of Nrg/ErbB signalling (Fig. 5) [27]. Similarly, cardiac contraction is necessary for the expression of Notch pathway elements like *notch1b* in the endocardium (Fig. 5) [29]. The Hippo pathway and in particular its effector Yap1/Wwtr1 has been described to translocate to the nucleus in response to cardiac function mechanical forces [105]. Wwtr1 is essential in the compact myocardium to maintain the compact wall architecture supporting trabeculation by controlling myocardial Notch signalling [105]. Furthermore, Wwtr1 is controlled by Nrg signalling (Fig. 5). Interestingly, Nrg, Notch, Yap1/Wwtr1 and cardiac function have all been shown to regulate the re-localisation of N-cadherin, a critical factor for trabecular CM delamination, highlighting the critical role of biomechanical forces in the regulation of trabecular initiation in zebrafish (Fig. 5) [26,27,30, 105].

Klf2, a member of the Krüppel-like family of transcription factors, is a well-known mechanosensitive gene with important roles in heart development in response to blood flow WSS [106,107]. Endocardial *klf2a* has been described to control chamber specification and valve development controlled by fluid forces. Its role in zebrafish cardiac trabeculation is to maintain the integrity of the CM compact layer by preventing CMs from extruding from the ventricular wall (Fig. 5) [31, 100]. In addition, several studies have described the critical role of cerebral cavernous malformation (CCM) family proteins including the transmembrane protein Heg1 as mechanosensors able to transduce mechanical signals intracellularly and control ECM dynamics upstream of Klf2 and Notch both in mice and zebrafish (Fig. 5) [41,92]. Finally, recent studies have also identified the role of biomechanical forces in the control of cardiac jelly distribution in zebrafish hearts [108]. *timp2b*, an inhibitor of metalloprotease activity critical for ECM degradation, is upregulated in the absence of cardiac function suggesting that ECM remodelling is also controlled by mechanical forces (Fig. 5) [108].

In contrast to the detailed morphological and molecular knowledge gathered in the zebrafish model, the knowledge in other model

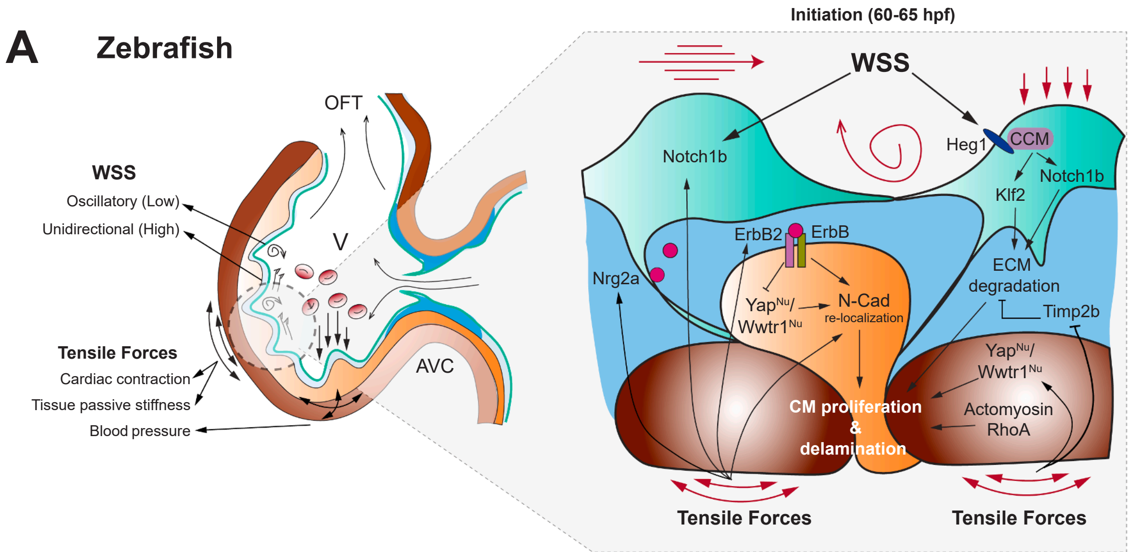

Fig. 5. Biomechanical regulation of cardiac trabeculation. (A) Biomechanical forces affecting heart development and the mechanotransduction pathways involved. Wall shear stress (WSS), Outflow Tract (OFT), Ventricle (V), Atrioventricular Canal (AVC), Cardiomyocyte (CM), Extracellular matrix (ECM).

organisms is less developed. This knowledge is mainly based on the morphological consequences of abnormal haemodynamics or cardiac contraction during heart development, and mostly referring to the growth and patterning of the trabecular network [3,4,34,109–112]. Indeed, studies in chicken have identified that trabecular patterning respond to hemodynamic changes, with cardiac flow reduction in chicken hearts leading to the hypoplasia of the left cardiac structures but an over development of the right cardiac structures, whereas pressure overload led to the thickening and dilation of the ventricular chambers [17,113,114]. The analysis of the well-known flow responsive genes ET1 (low shear stress), KLF2 and NOS3 (high shear stress) in the early chicken embryo defined the localization of the low and high shear stress areas in the developing heart [109]. However, very little is known about the molecular regulation downstream of these biomechanical forces in the control of cardiac trabeculation in chicken. Analysis of xenopus mutants for *myh6*, encoding for the α-MHC sarcomere protein, and lacking cardiac contraction, identified that most of the cardiac morphogenetic processes including primitive cardiac tube assembly, cardiac looping and chamber specification were relatively unaffected by the lack of biomechanical forces. However, cardiac defects associated with the processes of valve development and cardiac trabeculation were identified under these abnormal biomechanical conditions [34]. Similarly, morphological analysis of mouse mutants for the sarcomere proteins *Myl7* and *Tnnt2*, showing abnormal cardiac contraction, or WT embryos cultured under reduced hemodynamic loading, confirmed that the early cardiac morphogenetic processes leading to the looped heart were mostly unaffected, with clear defects associated to valve development and cardiac trabeculation [110–112]. However, the advances in our understanding on the role of endocardial and myocardial behaviours and ECM dynamics as well as the molecular regulation controlling cardiac trabeculation in recent years warrants a more detailed analysis of these and other models to determine the specific role of biomechanical forces during cardiac trabeculation and the molecular regulation controlling their mechanotransduction in these species. Of note, all vertebrate hearts start their development as a tubular heart. However, while the adult zebrafish forms a 2-chamber heart, frogs and reptiles develop a 3-chamber heart, and mammals and birds a 4-chamber heart. Therefore, these studies are especially important to better understand the potential differences in the role of mechanical forces during heart development as heart complexity increases.

Importantly, the study of biomechanical forces in mammalian models is especially complex as embryos grow inside the maternal uterus and require proper and constant blood circulation between the mother and the embryo to supply oxygen and nutrients [115]. Thus, these studies need to be mindful about the fact that blockage or reduction of blood flow in mammalian embryos may promote abnormal oxygen and nourishment levels potentially causing developmental defects not directly related to abnormal biomechanical forces. Indeed, both hypoxia and lack of nutrients have been described to cause heart defects during embryonic development [116–118]. Therefore, future research aiming to study these aspects will need to discriminate between the specific defects associated with biomechanical forces and secondary factors - such as hypoxia or nutrient deficiency - when analysing mammalian models with abnormal cardiac function.

2. Conclusions and Future Directions

Cardiac trabeculation is one of the most important processes during the formation and maturation of the ventricular chambers. Although our understanding of the process has significantly improved in the last decades, there are still important gaps in the current knowledge. In fact, studies on cardiac trabeculation in different vertebrate models have identified significant differences in the way the process occurs and how it is regulated at the molecular level putting in doubt its evolutionary conservation across vertebrates.

The most controversial phase is trabecular initiation. Although this phase has been deeply investigated in zebrafish and there is a significant level of understanding on the morphogenetic and molecular regulation of the process, most of this regulation have not been identified yet in other vertebrates. The studies on mouse, frog, chicken and human cardiac development referenced here suggest that either these processes take place before the formation of the primitive heart tube, or trabecular initiation occurs by different morphogenetic and molecular processes in these species. A detailed 3D characterization of heart development from the cardiac field stage to the heart tube formation in mouse, frog, chicken and human embryos may shed some light on this controversy.

Similarly, the role of endocardial behaviours controlling ECM dynamics, which is crucial during trabecular development in most vertebrate species, seems to be dispensable for zebrafish trabeculation. However, if we look at the earlier processes taking place before the delamination of CMs from the ventricular wall, we may find a solution for these discrepancies. At these earlier stages in zebrafish, cardiac jelly is present in the ventricular chamber and it is segmented into individual ECM bubbles by the endocardial subcellular protrusions embracing the delaminating CMs [31,39]. This process seems to be similar to endocardial sprouting and touchdown/dome formation in mice but at the cellular level (Fig. 2). Furthermore, at these earlier stages Notch1 activity is present in the endocardium regulating similar molecular pathways described in mice [12,14,29]. Therefore, a more detailed analysis in these early stages of zebrafish development may confirm the possibility that the process of trabecular initiation starts earlier, and it is controlled by the same endocardial and ECM dynamics controlling trabeculation in mice. However, even if we confirm these similarities, unless we identify CM delamination from the myocardial wall before the formation of the primitive heart tube in higher vertebrates, it seems evident that the process of trabecular CM formation may take place by different morphogenetic mechanisms between fish and other vertebrates. However, further studies are needed to confirm this possibility.

The different ECM dynamics taking place between zebrafish and other vertebrate species and their implication for trabecular development strongly suggest additional functions for the ventricular ECM in higher vertebrates that may be associated with other aspects of ventricular morphogenesis and potentially related to the development of more complex multichambered hearts. This also highlights the importance of investigating the role of biomechanical forces in other vertebrate species to identify their potential role during heart development as heart complexity increases. Likewise, it will be important to investigate whether endocardial sprouting is controlled by the same molecular pathways regulating tip/stalk cell decisions during endothelial sprouting angiogenesis. The control of sprouting angiogenesis is a finely regulated process that precisely determines the patterning of the new forming vessels. If proven, this will provide a robust patterning mechanism controlling the organization of endocardial touchdowns and as a result, the location and organization of the trabecular myocardium.

Another important aspect that warrants further research is how the trabeculation process is induced in specific regions of the ventricular chamber and if the process of trabecular initiation is stochastic, or instead genetically or epigenetically encoded. Also essential is how the patterning and organization of the trabecular myocardium is achieved and how reproducible the trabecular network is between embryos. The answers for these questions have been difficult to resolve as most of the analysis of trabecular development performed to date were done on tissue or optical sections, hence missing the 3D complexity of the trabecular network. However, with the improvement of tissue clearing methods and microscopy techniques together with the evolution of 3D image analysis methods, we are now better prepared to perform 3D reconstruction analysis and tissue structure comparison between embryos to answer these questions. Additionally, the development of single cell RNAseq and ATACseq methods have provided a huge improvement in our understanding of cell differentiation states or the predisposition of cells to respond more efficiently to molecular cues inside a given tissue. However, these techniques lack the tissue context to be able to precisely locate each cell inside the cellular complexity of a tissue like the trabecular myocardium. Therefore, only in combination with the recent advancements on spatial transcriptomics methods we will be able to perform robust analysis to answer these questions.

Finally, in the last decades, our understanding of the morphogenetic processes and molecular regulation controlling the formation of trabecular myocardium have advanced substantially. However, even though similar research efforts have been applied to the study of trabecular maturation and compaction, most of these studies have focused on the description of cardiac defects similar to human NCC and LVNC conditions associated with the dysregulation of specific genes and have not investigated in detail the morphogenetic processes controlling trabecular compaction, or the way trabeculae contribute to the formation of papillary muscles, interventricular septum or the ventricular conduction system. Therefore, further research aiming to identify the tissue and cellular behaviours, ECM dynamics and molecular regulation taking place during the process of trabecular myocardium maturation and compaction will be essential to improve our understanding of this aspect of trabeculation. This knowledge will be also crucial to pinpoint the specific morphogenetic processes that when dysregulated lead to NCC, LVNC and other congenital heart diseases promoting the identification of specific genetic determinants for these conditions and potential therapeutic approaches to improve patient outcomes.

Declaration of Competing Interest

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