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SPOTLIGHT REVIEW

Modulation of conductive elements by Pitx2 and their impact on atrial arrhythmogenesis

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Abstract The development of the heart is a complex process during which different cell types progressively contribute to shape a four-chambered pumping organ. Over the last decades, our understanding of the specification and transcriptional regulation of cardiac development has been greatly augmented as has our understanding of the functional bases of cardiac electrophysiology during embryogenesis. The nascent heart gradually acquires distinct cellular and functional characteristics, such as the formation of contractile structures, the development of conductive capabilities, and soon thereafter the co-ordinated conduction of the electrical impulse, in order to fulfil its functional properties. Over the last decade, we have learnt about the consequences of impairing cardiac morphogenesis, which in many cases leads to congenital heart defects; however, we are not yet aware of the consequences of impairing electrical function during cardiogenesis. The most prevalent cardiac arrhythmia is atrial fibrillation (AF), although its genetic aetiology remains rather elusive. Recent genome-wide association studies have identified several genetic variants highly associated with AF. Among them are genetic variants located on chromosome 4q25 adjacent to PITX2, a transcription factor known to play a critical role in left–right asymmetry and cardiogenesis. Here, we review new insights into the cellular and molecular links between PITX2 and AF.

Keywords Pitx2 • Cardiac development • Atrial fibrillation

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1. Developmental biology of the heart; from structure to function

The development of the heart is a complex process during which several types of cells progressively contribute to shape a four-chambered pumping organ. The first set of cardiac progenitor cells is symmetrically laid down soon after gastrulation to form the cardiac crescent.[1] These cardiac progenitor cells give rise to a linear heart tube that subsequently loops to the right and forms distinct atrial and ventricular cardiac chambers.[2] Two pools of genetically distinct cells[3–6] contribute to the embryonic heart: the first heart field (FHF) mainly contributes to the left ventricle, whereas the second heart field (SHF) gives rise to the rest of the heart.[7] A third population of cells will form the sinus venosus, potentially identifying a third heart field.[8] Subsequently, septation of each of the embryonic chambers as well as their connecting components results in the formation of a four-chambered heart with distinct systemic and pulmonary circulation.[2]

Over the last decades, our understanding of the specification and transcriptional regulation of cardiac development has greatly increased. Seminal papers in different experimental models such as zebrafish, chick, and mice have unravelled critical roles of Fgf and Bmp signalling in cardiomyocyte specification.[9–11] Soon thereafter, key transcriptional regulators such as Nkx2.5, Gata, and Mef2 family members are required from early stages of heart formation.[12–14] Subsequently, Hand genes play central roles in the establishment of systemic (left) and pulmonary (right) ventricular chambers,[15] and Tbx5 is required for correct interventricular septum development.[16] In addition to the mechanisms regulating intrinsic components of the developing heart, we have also deciphered key elements concerning proepicardium formation[17,18] and the contribution of cardiac neural crest cells to the developing heart.[19]

Our knowledge of developmental mechanisms underlying cardiac morphogenesis has progressively advanced as new technological tools have been discovered. Classical anatomical and morphological descriptions provided the founding bases to link developmental anatomy to congenital heart diseases.[20,21] The advent of molecular biology provided a new step forwards linking (dys)morphogenesis of the heart with genetic causes of congenital and adult heart diseases.[22] However, our understanding of the functional bases of cardiac

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electrophysiology during embryogenesis has remained largely unexplored, as has the link between developmental biology and cardiac physiopathology. Over the last few years, new links have been bridged, which will constitute the main theme of this review.

2. The development of a pumping heart: insights into its electrophysiology

In mice, as in man, the heart is the first organ to develop during embryogenesis and is the only organ functionally required throughout development. The nascent heart progressively acquires distinct cellular and molecular characteristics in order to fulfil its functional properties, such as the formation of contractile structures, the development of conductive capabilities, and soon thereafter the co-ordinated conduction of the electrical impulse. Conductive capabilities can be subdivided into those providing cell-to-cell wiring (such as gap junctions) and those conducting electrical stimuli, i.e. generating the cardiac action potential (such as ion channels). Finally, the electrical impulse is co-ordinated through the development of a specialized cardiomyocyte network termed the cardiac conduction system.

Cardiomyocyte sarcomere formation is a key step in providing contractile function to the heart. Sarcomere formation is a complex process, which is nonetheless highly conserved across species, involving the progressive establishment of actin–myosin myoarchitecture, including the troponin–tropomyosin complex, in addition to cytoskeletal coupling. Expression of sarcomeric proteins is one of the earliest events in murine cardiogenesis.[23] The main sarcomeric components, such as myosin heavy (*Myh*) and light (*Myl*) chains, and actin and troponin isoforms are expressed in the cardiac crescent.[24,25] Soon thereafter, *Myh*s and *Myl*s display atrial (*Myh6*, *Myl4, Mly7*) or ventricular (*Myh7*, *Myl2, Mly3)* chamber-specific expression profiles, whereas other components of the sarcomere, such as actins and troponins are more broadly expressed in the developing myocardium. Interestingly, isoform-specific replacement within distinct cardiac domains is progressive, observed for several sarcomere components.[26] For example, smooth (*Acta2*) and skeletal muscle actin (*Acta1*) are expressed during embryonic (E9.5–14.5) stages, but from foetal stages (E15.5 onwards) only cardiac actin (*Actc1*) is present in the developing heart. Thus, nascent cardiomyocytes are already capable of contracting (*Figure 1*) and distinct functional capabilities are rapidly established within the different compartments of the forming heart.[26]

Conductive capabilities permitting cell-to-cell wiring, i.e. gap junctions, have also been extensively studied in mice and rats. Gap junctions are mainly formed by connexons, which are in turn made of connexins.[27] Several types of connexins are expressed in the developing and adult heart, including connexin30.2 (Cx30.2), connexin40 (Cx40), connexin43 (Cx43), and connexin45 (Cx45), and display regional heterogeneity.[27–29] Importantly, each connexin has distinct functional capacities; for example, Cx40 and Cx43 display high conductance, whereas Cx30.2 and Cx45 display low conduction.[30] Connexin30.2 (*Gjd3*) is expressed essentially in nodal cells, connexin40 (*Gja5*) in atrial myocardium as well as the fast tracts of the ventricular conduction system, whereas connexin43 (*Gja1*) is expressed in both atrial and ventricular myocardium but excluded from the conduction system.[30] The expression profile of connexin45 (*Gja7*) remains rather controversial since some authors have provided evidence of a highly dynamic expression during cardiac development, with overt expression in the early looping heart (E8.5), becoming progressively to be confined to the atrial chambers,[31] whereas others have described a pattern mainly restricted to the developing cardiac conduction system.[32] In contrast to the contractile elements, gap junctional proteins are not detected in cardiomyocytes of the cardiac crescent or early heart tube. The first expression of connexins (Cx45) is reported in the looping heart, becoming more robust (Cx40 and Cx43) at later embryonic stages [embryonic day (E) 13.5 in rats or E11.5 in mice] when atrial and ventricular chambers are forming.[28] Thus, co-ordinated cell-to-cell conduction is established during development after cardiomyocytes acquire contractile properties, providing a further layer of functional complexity to the different emerging cardiac compartments. Moreover, cell-to-cell conduction evolves from a random distribution of connexins over the cell surface into a highly organized and restricted surface distribution at intercalated discs[28,33] (D. Franco and J.N. Dominguez, unpublished data), thus providing a putative means to change from isotropic to unidirectional and anisotropic conductance (*Figure 1*). It remains to be established how cell-to-cell conduction is achieved in the early heart tube.

In addition, conductive capabilities of the pumping heart are also promoted as a response to electrical stimuli by the generation of action potentials.[34] The generation and regulation of the cardiac action potential is exquisitely complex. Taking as a reference the cardiac action potential of the adult human heart, at least five phases can be distinguished in which multiple ionic currents participate.[34] The first phase (Phase 0) in atrial and ventricular myocytes is mediated by a sodium current through voltage-gated Na^+ channels (I_{Na}) which leads to cell depolarization. Initial rapid repolarization (Phase 1) is the consequence of the rapid voltage-dependent inactivation of I_{Na} and the activation of two rapidly activating outward K currents, the transient outward K^+ current (I_{to1}), and the ultrarapid component of the delayed rectifier current (I_{Kur}). In humans, I_{Kur} is recorded in atrial but not in ventricular cells. During Phase 2 (plateau phase), inward depolarizing currents through Na^+ (slowly inactivated) and L-type Ca^{2+} channels (I_{CaL}) are balanced by the ultrarapid, rapid, and slow components of the delayed rectifier K^+ current (I_{Kur}, I_{Kr}, and I_{Ks}). The influx of calcium ions provided during this phase is responsible for the excitation–contraction coupling. During the final phase of repolarization (Phase 3), Na^+ and Ca^{2+} channels close, while the channels which generate the delayed rectifier currents (I_{Kur}, I_{Kr}, I_{Ks}) are still open. This ensures a net outward current that repolarizes the cell, allowing the opening of the inward rectifying K^+ currents, mainly I_{K1}, the ATP-sensitive K^+ current (I_{KATP}), and the acetylcholine-activated current (I_{KACh}), especially in the atria. These inward currents are also responsible for the maintenance of the resting membrane potential during Phase 4. Whereas distinct phases of the action potential configuration are registered in vertebrate species, such as in pigs, rats, mice, and humans, important differences on the contribution of each current are observed, thus providing distinct action potential phenotypes. In addition, importantly, and similarly to the regional differences in the contractile and cell-to-cell conductive properties of the adult cardiomyocytes, the shape of the cardiac action potential greatly differs between atrial, ventricular, and nodal cells (*Figure 1*). In working atrial and ventricular cardiomyocytes, the upstroke phase of the cardiac action potential is driven by the I_{Na}, whereas the diastolic

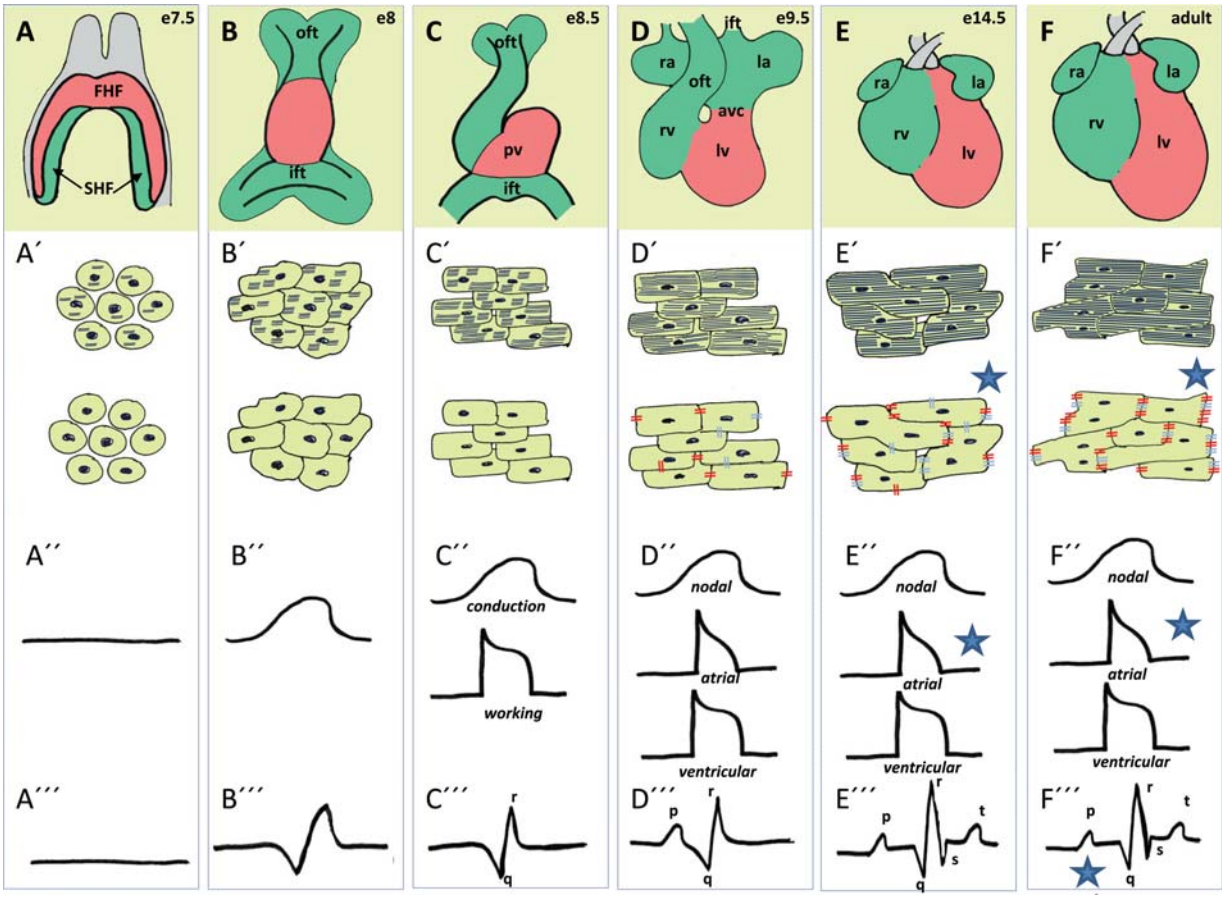

Figure 1 Schematic illustration of progressive contractile and conductive maturation during cardiogenesis. Schematic representation of the different stages of mouse cardiac development: cardiac crescent (*A*) straight tube, (*B*) looping, (*C*) embryonic, (*D*) foetal, (*E*), and adult (*F*) heart. The progressive formation of contractile and cell-to-cell conductive elements is depicted in (*A′*)–(*F′*). Cell-to-cell wiring illustrates the progressive alignment of connexins during cardiomyocyte differentiation. Similarly, progressive development of the cardiac action potential and the ECG recordings are illustrated in (*A″*)–(*F″*) and (*A‴*)–(*F‴*), respectively. Cardiac action potential diversity between conductive and working myocardium is illustrated at embryonic (E8.5) stages and between nodal, atrial, and ventricular myocytes from late embryonic stages (E9.5) onwards. Note that while cardiac action potential configuration resembles that of man, yet significant differences are observed in other species. Asterisks demarcate those stages and pathways in which impaired expression and/or function has been documented on Pitx2 deficiency. FHF, first heart field; SHF, second heart field; oft, outflow; ift, inflow; pv, primitive ventricle; rv, right ventricle; ra, right atrium; lv, left ventricle; la, left atrium; avc, atrioventricular canal.

potential is constant due to the inward rectifying currents. In contrast, in nodal cells, the upstroke is mainly governed by calcium entry to the cell, whereas the diastolic potential which is not constant, progressively increases towards the threshold potential as a consequence of the activation of the pacemaker current (funny current, I_f).[34]

Gene expression analyses of components involved in generating the cardiac action potential reveal that the earliest expression of sodium, potassium, and calcium channels is mainly observed soon after the heart has looped.[33–37] Importantly, distinct ion channel expression profiles are observed in atrial vs. ventricular chambers.[36] Seminal work shedding light on the early electrophysiological characteristics of the developing mouse heart was reported by Davies *et al.*[38] These authors explored the establishment of action potential currents during cardiomyogenesis, demonstrating a similar basal I_{Na} current in atrial and ventricular myocytes at embryonic (E11.5) stages, which progressively peak at late foetal (E17.5) stages, similar to the situation during chick[39] and rat[40] cardiogenesis. These observations are consistent with the observation of a progressive increase in Scn5a/Nav1.5 and Scn1b expression during mouse cardiogenesis.[33,35] Similarly, Davies *et al.*[38] demonstrated that some (I_{to}, I_{Kr}), but not all (I_{Ks}), repolarizing ion currents, are already functionally operative during embryonic (E11.5) and foetal (E17.5) stages of cardiac development, as suggested by gene expression analyses.[38] Importantly, I_{to} was more prominent in atrial compared with ventricular cardiomyocytes during mouse embryogenesis, whereas I_{Kr} only displays an atrial predominance in embryonic (E11.5) but not foetal (E17.5) stages during mouse cardiogenesis. However, the inward rectifier potassium current (I_{K1}), which is responsible for the maintenance of the resting membrane potential, is only detectable at foetal stages with similar levels in atrial and ventricular cardiomyocytes. Surprisingly, embryonic cardiomyocytes display high levels of ATP-regulated potassium (I_{KATP}) channels, in marked contrast to the adult situation,[38] suggesting a predominant role for I_{KATP} currents during mouse cardiogenesis. Calcium currents (I_{Ca}, L-type) are also operative at embryonic (E11.5) and foetal (E17.5) stages, thus providing a means to couple excitation and contraction at early developmental stages, as reported

by Davies et al.,[38] consistent with gene expression[37] and functional evidence[41] demonstrating that several components of the excitation–contraction coupling system are present at early developmental stages. Interestingly, L-type calcium currents (I_{Ca}) are more prominent than sodium currents (I_{Na}) at embryonic stages, raising the possibility that, besides their role in excitation–contraction coupling, these currents might also exert a pivotal role as excitatory currents during early mouse heart development. Together, these data suggest that the conductive properties of the early heart are significantly different from those at later stages (*Figure 1*).

The integration of conduction and contraction results in the generation of a contractile wave within the heart. Initial analyses of how the heart develops its contractile properties were carried out in the chick model, owing to its accessibility and versatility to be recorded under normal as well as experimental conditions. At the linear heart tube stage, the heart beat exerts a peristaltic contraction with an anteroposterior polarity,[42,43] revealing the presence of a dominant pacemaker activity at the posterior, or venous, pole of the heart.[44,45] At this stage, no specialized cardiac conduction system can be morphologically identified and how early pacemaker activity is generated remains to be elucidated.

Synchronous contraction is developed as the heart loops and forms distinct atrial and ventricular chambers, together with discrete atrioventricular (AV) and outflow tract regions. At this stage, the cardiac conduction system still cannot be morphologically and/or functionally traced. In fact, sinoatrial pacemaker activity fluctuates from the left to the right side at this stage.[45] Finally, as the heart acquires a four-chambered configuration, establishment of the cardiac conduction system results in an apical-to-base ventricular conduction pattern, with an electrocardiogram (ECG) recording similar to adult stages (*Figure 1*). Although experimental work in chick has provided enormous understanding of cardiac electrophysiology during embryogenesis, the fact that the chick conduction system displays diffuse and poorly recognizable nodes has hampered extrapolation of these findings to the mammalian heart, and thus to man.

Over the last few years, technological advances have provided the means to explore the electrophysiological properties of the developing heart in mice. The combination of transgenic mice that specifically delimit the forming cardiac conduction system and methodological approaches that exquisitely map the electrophysiological features of the forming heart[46,47] has provided a rather accurate picture of the electrophysiological characteristics of the developing mouse heart, providing a closer approximation of the human situation. Such technological advances have revealed that peristaltic contraction in the early linear heart tube stage is followed by synchronous contraction as the atrial and ventricular chambers are established in mice, while apex-to-base ventricular conduction is established just before and/or concomitantly with morphological delimitation of the ventricular conduction system.[47] Nonetheless, the definitive ventricular activation pattern is only established at late foetal stages in mice as reported by Sedmera et al.[48,49] In addition, we have also advanced our understanding of the origin of the cardiac conduction system, particularly the cellular origin of the ventricular conduction system[50,51] as well as the development of the sinoatrial and AV nodes.[52]

Thus, the developing heart progressively acquires the capabilities for contracting and conducting electrical impulses in a synchronous and co-ordinated fashion. However, while much is now known about how impaired cardiac morphogenesis leads to congenital heart defects, the consequences of impaired electrical function during cardiogenesis, and the consequences of such defects on the definitive heart, remain unknown.

3. Developmental genetics of atrial arrhythmias

Cardiac arrhythmia can be defined as a variation from the normal heart rate and/or rhythm that is not physiologically justified, resulting in a specific ECG pattern. Atrial arrhythmias may be regular, as in the case of flutter or monomorphic tachycardia, or irregular, as in the case of fibrillation or polymorphic tachycardia. Some rhythm disturbances may be relatively benign, as in the case of premature ventricular contractions, whereas others are malignant and can result in sudden death.[53] Here, we focus on atrial fibrillation (AF), the most prevalent type of arrhythmia in humans.

AF is a supraventricular tachyarrhythmia characterized by uncoordinated atrial activation with consequent deterioration of atrial mechanical function. In AF, P-waves are replaced by rapid oscillations or fibrillatory waves that vary in size, shape, and timing, and are associated with an irregular, frequently rapid ventricular response when AV conduction is intact.[54] The prevalence of AF is highly dependent on age, ranging from 1% in young adults to ~10% of those over 80 years old.[55] AF is frequently associated with diverse cardiac or systemic disorders including hypertension, coronary disease, valvular diseases, and cardiomyopathies.[56] However, in 10–20% of AF patients, no underlying disease or precipitating factors can be found,[57] and such cases are termed 'lone or idiopathic AF'.

Great insights have been gained concerning the cellular and molecular mechanisms that underlie AF. In this context, two main hypotheses have been explored. First, the multiple-wavelet hypothesis[58] proposes that wave fronts are fractionated as they propagate within the atria, thus resulting in derived wavelets that self-perpetuate. The number of wavelets observed at any time depends on the conduction velocity, mass, and refractory period within the distinct regions of the atria. Although the activation patterns leading to irregular atrial electrical activity in AF have conventionally been described as random or disorganized, recent evidence has emerged supporting that AF might be spatially organized. The second hypothesis suggests that enhanced automaticity in one or several rapidly depolarizing foci and re-entry involving one or more circuits[59] leads to AF. These foci are frequently spotted as one or several foci within the superior pulmonary veins, and more infrequently within the superior vena cava or coronary sinus.[60]

Although AF is the most prevalent type of arrhythmia, its genetic aetiology remains elusive. Several reports have documented familial cases,[58,61] providing impetus to search for the genetic bases of AF. The first genetic locus for AF was reported in 1997 by Brugada et al.[62] identifying linkage to chromosome 10q22–q24. A second locus was later identified by Chen et al.[63] which identified linkage in a single family with AF to chromosome 11p15, and a mutation in *KCNQ1* was demonstrated using a candidate gene screening approach. *KCNQ1* mutations in AF have been subsequently confirmed in other studies.[64] Since then, mutations in several ion channels including *KCNE2*, *KCNE3*, *KCNJ2*, *KCNA5*, and *SCN5A* have been described,[65–68] although their frequency is <1% in AF patients. Similarly, mutations in lamin A/C (*LMNA*) have also been shown to cause AF in patients, and families with dilated cardiomyopathy and conduction disease[69]

or somatic mutations in *GJA5*[70] are prone to AF but again the incidence is low.

The advent of new genetic strategies, in particular genome-wide association studies (GWAS), has shed new light on the genetic bases of arrhythmogenic syndromes and the identification of novel disease-causing genes.[71] This approach involves large scans of the genome using a dense set of SNPs in order to identify causal variants.[72] GWAS have identified several genetic variants highly associated with lone AF, which are located at 9p21, 1q21, 16q22, and 4q25, respectively. On chromosome 9p21, no candidate gene has been linked to AF, and thus its relevance awaits further experimental and functional evidence.[71] Genetic variants at 1q21 are linked to *KCNN3*, a potassium channel involved in atrial repolarization,[73] and genetic variants at 16q22 are linked to *ZFHX3*, a zinc finger homeobox transcription factor involved in liver gene expression,[74] the role of which in AF remains obscure. Finally, genetic variants on chromosome 4q25 which are strongly associated with lone AF in three distinct populations of European descent,[75] map adjacent to *PITX2*, which is known to have a critical function in establishing left–right asymmetry of the heart,[76,77] and plays a critical role in the development of the left atrium,[78–81] as well as in the development of pulmonary vein myocardium.[81] Thus, Gudbjartsson *et al.*[75] hypothesize that impaired function of *PITX2* might underlie AF.

4. The role of PITX2 in AF

Pitx2 is a homeobox transcription factor involved in left–right signalling during embryogenesis.[76,77] Disruption of left–right signalling in mice within either its core *nodal*/*lefty* cascade[82–84] or within other less conserved players such as the ion channel Pkd2[85] results in impaired expression of the last effector of the left–right cascade, Pitx2, leading in many cases to bilateral expression of *Pitx2* in lateral plate mesoderm (LPM). Loss of *Pitx2* expression in LPM results in severe cardiac malformations, including right cardiac isomerism.[82–85] However, whereas the pivotal role of Pitx2 during cardiogenesis is well sustained, its putative role in the foetal and adult heart is largely unexplored. The Pitx2 gene encodes three distinct isoforms, *Pitx2a*, *Pitx2b*, and *Pitx2c*, through alternative splicing and distinct promoter usage.[86] In the developing mouse heart, Pitx2c is the most prominent isoform. Only weak and transient expression of *Pitx2a* is observed during early embryonic (E9.5–E12.5) stages, whereas *Pitx2b* displays progressively decreasing expression in embryonic and foetal stages (D. Franco and A. Aranega, unpublished data). In humans, a fourth isoform, *PITX2D* is also expressed, which can act as a dominant negative protein.[87] Mutations in *PITX2* are associated with Axenfeld-Rieger syndrome, characterized by abnormal morphogenesis of the anterior segment of the eye, and a variable degree of maxillary hypoplasia, skeletal abnormalities, and abdominal defects.[88]

Pitx2 displays a highly dynamic expression profile during cardiogenesis. Pitx2 is firstly expressed asymmetrically in the left but not right LPM, before the cardiac crescent forms, and subsequently, as the heart develops, becomes confined to the left side of the linear heart tube.[78–80] Expression of Pitx2 is remodelled during cardiac looping, becoming localized to the ventral portion of the developing ventricular chambers, while maintaining a distinct left-sided atrial expression.[79] With further development, Pitx2 is down-regulated in the ventricular chambers, while high and robust expression of Pitx2 is maintained in the atrial chambers as well as in discrete components of the inflow tract, including the left superior caval vein, the pulmonary veins, and the interatrial septum.[89] Importantly, left–right differential expression of Pitx2 is also observed at the arterial pole.[79] Such a dynamic expression profile results in Pitx2 expression within both first and second heart fields during cardiogenesis.[90]

The importance of Pitx2 during cardiogenesis has been illustrated by the complex and robust cardiac defects observed on systemic deletion of Pitx2 in mice. Lack of Pitx2 expression leads to embryonic lethality at mid-term, and Pitx2-deficient embryos display isomeric hearts and incomplete closure of the body wall.[76,77,91,92] Conditional mutants have unraveled detailed information as to the role of Pitx2 during cardiogenesis. Using conditional tissue-specific Cre deletor mice, Ai *et al.*[93] revealed that eliminating Pitx2 function in the second heart field leads to developmental defects reminiscent of systemic Pitx2c null mutants.[91,92] Further evidence on the role of Pitx2 within the secondary heart field has been reported at the arterial[94,95] and venous[96] poles of the heart. In this context, it is important to note that Pitx2 exerts an asymmetric repressive effect in posterior secondary heart field explants,[96] and over-expression in embryonic stem cell-derived cardiomyocytes leads to transcriptional up-regulation of the SHF marker *Isl1*, whereas expression of *Nkx2.5* is unaltered.[97] Thus, there is strong evidence that Pitx2 functions in SHF derivates including structures prone to provoke AF at the venous pole of the heart.

Importantly, it has been reported that Pitx2 function is not exclusive to cardiac precursor cells but is also relevant in differentiated cardiomyocytes. Tessari *et al.*[98] have generated conditional Pitx2 null mutants in cardiomyocytes using αMHC-Cre driver mice, and demonstrated that functional impairment of Pitx2 in cardiomyocytes leads to cytoarchitectural disarray in ventricular myocytes and to abnormal left–right expression of *Bmp10* in the atria. Surprisingly, αMHC-CrePitx2 mutants do not display atrial isomerism demonstrating a clear difference with previous systemic Pitx2 mutants.[76,77,91,92] Thus, while Pitx2 plays a crucial role in fully differentiated cardiomyocytes, left/right atrial morphological identity is acquired before αMHC-Cre activation (E8.5–9.0 in the atrial chambers). Moreover, these data demonstrate a very early developmental role of Pitx2 in venous pole structures prone to triggering atrial arrhythmias.

If we focus on the venous pole of the heart, the contribution of Pitx2 is highly complex, probably for two reasons. First, because the morphogenesis of this heart is relatively poorly understood and, secondly, because the lack of appropriate molecular tools to finely dissect the distinct cellular and molecular components of the venous pole has hampered analysis of gene function within these specific structures. Asymmetric expression of Pitx2 within the developing inflow tract was reported several years ago, discretely differentiating left and right components in both the systemic and venous components of the inflow tract.[90] These data suggested therefore an essential role of left/right signalling in controlling venous pole development. Consistent with this hypothesis, detailed analyses of systemic Pitx2 mutant mice revealed impaired pulmonary myocardial formation in right atrial isomeric hearts.[86,99] Although it has been clearly established that Pitx2 plays a role in pulmonary vein development, a frequent foci of AF, we should note that such alterations occur in the setting of complex cardiac abnormalities such as atrial isomerism and, secondly, that pulmonary vein development differs between mice and men. In rodents, a single pulmonary orifice enters the left atrium, while in man there are four orifices with a surrounding pulmonary vein-derived myocardial region. Additional

evidence is thus required to fill the gap between Pitx2 function in the embryo and the onset of AF.

Genetic variants at 4q25, close to the Pitx2 locus, are highly associated with AF,[76] suggesting a role for PITX2 in the adult heart. In view of the developmental role of Pitx2 during cardiogenesis[100] and its involvement in pulmonary vein development,[99] these authors postulated that Pitx2 dysfunction might be a causative link for the onset of AF. AF has always been considered a left side disease and therefore implication of a left–right signalling gene, Pitx2, is highly concordant. However, there are several missing links. The first one is how the identified genetic variants alter Pitx2 expression. To date, it remains elusive how altered Pitx2 expression would modify conductive elements leading to the onset of AF. On the basis of the developmental role of Pitx2, it might be postulated that impaired Pitx2 in the developing heart might be a predisposing factor for AF. Alternatively, Pitx2 function may be exclusively impaired in the adult heart. Indeed, an early defect in Pitx2 function would be expected to have severe consequences for cardiac embryogenesis, and thus it is more likely that foetal or adult dysfunction might underlie Pitx2 predisposition to AF. Previous work in our laboratory using an Pitx2 over-expression model of embryonic stem cell-derived cardiomyocytes[97] as well as atrial and ventricular chamber-specific conditional Pitx2 mutant mice[101] has shown that loss of Pitx2 does not modify the expression of contractile proteins such as actin and myosin heavy and light-chain isoforms, thus suggesting that Pitx2 does not impair the contractile properties of cardiomyocytes. Importantly, Pitx2 mis-expression impairs connexin40 expression,[97,101] both in embryonic stem cell-derived cardiomyocytes (Pitx2 gain-of-function mice) and in foetal and adult atrial and ventricular chamber-specific Pitx2 loss-of-function mice. In agreement with these findings, Kirchhof *et al.*[102] have recently described impaired gap and tight junction expression in microarray analysis of the left atria of adult Pitx2 heterozygous mutant mice. These data provide a putative link to AF since mutations in GJA5 (Cx40) are associated with AF[70] and further implicate impaired Pitx2 function at foetal and/or adult stages in predisposition to AF.

In addition, Pitx2 might be modulating ion channel expression, and therein providing molecular substrates for atrial arrhythmogenesis. In this line of thinking, the action potential duration is shortened in the left atrial of Pitx2 heterozygous adult mutant mice, thus increasing their inducibility to AF.[102] Our recent work using atrial-specific conditional Pitx2 mouse mutants, in which right-sided sinoatrial node formation is intact, also supports a role of Pitx2 impairment on the onset of left-sided electrophysiological defects,[101] which are prone substrates for AF. Voltage-gated sodium and inward rectifying K^+ (Kir2.1, Kir2.2, and Kir2.3) ion channels are abnormally expressed in the atrial myocardium both in foetal and adult stages. As a consequence, in these mice, the action potential amplitude is decreased, while the resting membrane potential is depolarized. Curiously, *KCNN3*, which has recently been linked to AF by GWAS, is altered by *Pitx2* insufficiency, thus suggesting a dependent pathway leading to AF. Further evidence on the dysregulation of ion channels by Pitx2 impairment has been recently reported also by Kirchhof *et al.*[102] using mRNA microarray analyses in Pitx2 heterozygous adult mutant mice. Thus, these data reveal a role for Pitx2 controlling ion channel expression, and thus a link to atrial arrhythmogenesis (*Figure* 2).

Finally, Pitx2 might also trigger AF by altering the conduction properties of the cardiac conduction system. In this setting, Wang *et al.*[103] have demonstrated that Pitx2 haploinsufficiency predisposes to AF in electrically stimulated adult mice. These authors reported a developmental link in that Pitx2 inhibits the sinoatrial node gene programme in the embryonic left atrium, by regulating *Shox2* expression. When Pitx2 function is partially (heterozygous) or completely (null) impaired in the embryonic (E12.5) mouse heart, *Shox2*, as well as several other sino-atrial node markers such as *Hcn4* and *Tbx3*, are ectopically expressed in the forming inflow tract of the heart. These observations provide a link between the embryonic function of Pitx2 and AF (*Figure* 2).

5. Concluding remarks and perspective

We have highlighted herein our current knowledge of the development of the conductive properties of the forming heart, and recent evidence that Pitx2, implicated by genetic variants highly associated with AF, can lead to the onset of arrhythmogenic substrates. Several studies have shown that Pitx2 is essential for pulmonary vein development, a highly recurrent pro-arrhythmogenic region in humans. Experimental evidence has shown that Pitx2 is important for transcriptional regulation in the SHF, derivatives of which will form the venous pole of the heart. Similarly, additional evidence has

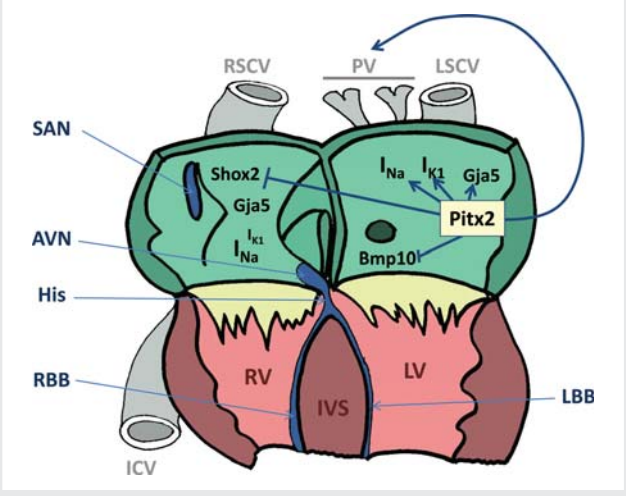

Figure 2 Schematic representation of the role of Pitx2 in atrial function. Schematic representation of the four-chamber adult heart illustrating the distinct signalling pathways modulated by the homeobox transcription factor Pitx2 relevant for atrial chamber function, and thus for atrial arrhythmogenesis. Pitx2 regulates connexin (Cx40; *Gja5*) expression, inward potassium (I_{K1}) and sodium (I_{Na}) currents, and Bmp10 expression in the developing foetal and adult left atria. Similarly, Pitx2 represses Shox2 expression in the left atrium of the embryonic heart. Such impaired expression of Shox2 would affect the electrophysiological characteristics of the pulmonary veins, a derivative of the embryonic inflow tract. Note that the inward potassium channel (I_{K1}) is more prominent in the left atrium than in the right atrium. RV, right ventricle; LV, left ventricle, IVS, interventricular septum; SAN, sinoatrial node; AVN, atrioventricular node; His, bundle of His; RBB, right bundle branch; LBB, left bundle branch; RSCV, right superior caval vein; LSCV, left superior caval vein; ICV, inferior caval vein; PV, pulmonary veins.

revealed a role of Pitx2 in the regulation of cell-to-cell communication proteins and essential ion channels, which modulate critical characteristics of the cardiac action potential. In addition, Pitx2 plays a role repressing the sinoatrial node gene programme. Overall, these data strongly implicate Pitx2 as an upstream regulator of pro-arrhythmogenic events in AF.

However, many questions are still open. For example, it remains to be established how the genetic variants on chromosome 4q25 influence Pitx2 expression and/or function. It also remains to be established if impaired Pitx2 function leading to AF is determined at embryonic stages of cardiac development, as suggested by Wang *et al.*,[103] or whether dysfunction might affect the role of Pitx2 in the foetal or adult heart, as suggested by Chinchilla *et al.*[101] and Kirchhof *et al.*[102] In addition, it is unclear if Pitx2 deficiency might also lead to impaired formation and/or function of other components of the ventricular cardiac conduction system such as the AV node and the left and right AV bundle branches. Morphological analysis of the ventricular conduction system in the left–right mutant mouse model *iv/iv* suggests that left/right cues can influence AV node formation.[104] Nonetheless, further studies are required to demonstrate a putative role of Pitx2 in ventricular conduction system deployment.

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