ANALYSIS OF THE ROLE OF THE HOMEBOX TRANSCRIPTION FACTOR PITX2 IN CARDIAC DEVELOPMENT:
A CONDITIONAL KO APPROACH

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SUMMARY

Pitx2 is a homeodomain transcription factor expressed during embryogenesis which is crucial for the development of many organs i.e. eyes, teeth, pituitary gland and heart (Gage et al., 1999). Additionally, Pitx2 acts as the downstream mediator of the left-right pathway for imparting and maintaining “leftness” during morphogenesis of the asymmetrical organs (Levin et al., 1995; Isaac et al., 1997; Logan et al., 1998; Ryan et al., 1998). Both these actions are exerted also in the heart, which is asymmetrically patterned and where the gene is expressed from organ primordium formation and throughout all cardiac development (Campione et al., 2001). Nevertheless, how Pitx2 carries out its morphogenetic action is unknown.

A good murine model to investigate the role of the gene during heart development is still missing so far. In order to dissect the myocardial role of Pitx2 we have generated a myocardial conditional Pitx2 ko mice by crossing Pitx2 floxed mice (Pitx2loxP/loxP) (Gage et al., 1999) with TropoT (TnT) Cre deleter mice, which display efficient myocardial Cre activity from early cardiomyogenesis (Jiao et al., 2003); in the cTP ko mice even the earliest myocardial expression of Pitx2 is prevented. The characterization of the cTP ko model is the object of this study; as the ko phenotype is not compatible with life, the work has been conducted on embryos.

Hystological and magnetic resonance imaging analysis of E14.5 mutant hearts have revealed a complex set of cardiac morphological abnormalities including the typical features of the right atrial isomerism (RAI), a pathological condition in which both the atrial chambers develop the anatomical features and the venous connections of the right one; in particular bilateral sinoatrial nodes (SANs) and symmetric atrial entrance of the systemic veins have been detected. The morphological analysis has been associated to an extensive molecular characterization mainly performed by nonradioactive in situ hybridization whole mount and on section to assess the possible transcriptional modulation of developmental and functional markers expressed with regional specificity. The presence of an ectopic, left SAN and the finding of a topologically altered expression of molecules involved in the development of ventricular conduction system components prompted us to investigate the electrophysiological properties of the embryonic hearts. We approached this issue by optical mapping technique in collaboration with Prof. David Sedmera (Institute of Animal Physiology and Genetics, Prague); this tool provides epicardial membrane potential recordings with high temporal and spatial resolution, giving an ex vivo physiological readout about impulse generation and propagation in the tissue. Loss of Pitx2 expression affected ventricular morphology less dramatically than the sinoatrial region, therefore a confocal analysis has been additionally performed to investigate the ventricular phenotype at the cellular level.

In the study here presented we found that:
1 cTP ko mice are a good model of RAI, since the absence of Pitx2 from the left sinoatrial region results in a bilateral “right” morphological phenotype. The cTP ko hearts recapitulate most of the abnormalities described in a previously characterized Pitx2 null model (Gage et al., 1999), including the atrial, isomorphic phenotype; this last feature was absent in a myocardial specific Pitx2 ko generated with α-MHC Cre driver mice (Tessari et al., 2008) and resulting in silencing of the gene one day later than in the cTP model. Overall, these results indicate that early Pitx2 expression in myocardial cells, and not in their mesenchymal precursors, is a necessary and sufficient condition to confer “left” molecular and morphological identity to the sinoatrial region.

2 Pitx2 modulates the developmental transcriptional properties of the myocardial sleeves covering the systemic and the pulmonary venous return: loss of Pitx2 in the venous system leads to the ectopic maintenance (in caval veins) or activation (in the pulmonary myocardium) of a nodal-type genetic program including Hcn4, a potassium channel potentially sufficient to provide automaticity (Stieber et al., 2003). Consistently, the electrophysiological properties of the atria and venous pole have been found to be dose-dependently modulated by Pitx2 expression. Therefore, Pitx2 acts not only by driving asymmetric morphogenesis of the heart, but also finely modulating the transcriptional properties of cardiac regions where it is expressed. This role can be exerted in association with the morphogenetic action or independently from it and might be potentially relevant in the light of recent findings suggesting a role for Pitx2 in atrial fibrillation (AF), pathophysiology (Gudbjartsson et al., 2007; Lubitz et al., 2010): in our opinion Pitx2 disregulation could trigger AF onset by inducing arrhythmogenic foci in caval veins and pulmonary myocardium.

3 Ventricular cTP ko myocardial cells are less elongated and have a reduced myofibrillar alignment compared to the wt; the result was not recapitulate in the atria. Therefore, Pitx2 seems to promote the cellular maturation of ventricular but not atrial cardiomyocytes; this action might drive the ventricular remodelling process which is defective in the cTP ko embryos.

Concluding, Pitx2 myocardial expression acts differentially to impart a “left” molecular, morphological and functional sinoatrial identity and to drive ventricular remodelling.
RIASSUNTO

Pitx2 è un fattore di trascrizione homeobox espresso durante l’embriogenesi e determinante per lo sviluppo di diversi organi tra cui gli occhi, i denti, la ghiandola pituitaria e il cuore (Gage et al., 1999). In aggiunta al suo ruolo morfogenetico, Pitx2 agisce a valle della cascata di attivazione del segnale che determina il conferimento dell’identità sinistra negli organi asimmetrici (Levin et al., 1995; Isaac et al., 1997; Logan et al., 1998; Ryan et al., 1998). Entrambe queste due azioni vengono svolte in maniera integrata durante lo sviluppo del cuore, organo asimmetrico nel quale il gene viene espresso fin dalla formazione del primordio del cuore e nel corso di tutto il suo successivo sviluppo (Campione et al., 2001). Sebbene sia chiara la centralità della funzione di Pitx2 nello sviluppo cardiaco, i meccanismi tramite i quali esso è in grado di dirigere la sua morfogenesi asimmetrica non sono stati compresi.

Ad oggi, non è ancora stato ancora descritto un buon modello murino per lo studio del gene nel contesto della morfogenesi cardiaca. Per discernerne il ruolo miocardico, abbiamo quindi generato un modello murino di ko condizionale incrociando una linea di topi con l’omeodominio di Pitx2 fiancheggiato da siti LoxP (Pitx2 \(\text{loxP/loxP}\) (Gage et al., 1999) e una linea esprimente la Cre ricombinasi sotto il controllo trascrizionale del promotore della troponina T cardiaca (TnT) (Jiao et al., 2003). I topi cTP ottenuti da questo incrocio mostrano un elevata attività della ricombinasi fin da fasi molto precoci dell’embriogenesi, che consente la completa inattivazione funzionale del gene già dai momenti iniziali della cardiogenesi. La caratterizzazione del modello cTP è stata oggetto del seguente studio; poiché i cTP ko hanno un fenotipo letale, il lavoro è stato condotto su embrioni.

L’analisi istologica e mediante risonanza magnetica di cuori mutanti E14.5 ha rivelato un fenotipo complesso, composto da una serie di gravi anomalie cardiache tra cui l’isomerismo atriale destro (IAD), una condizione patologica in cui entrambi gli altri si sviluppano secondo le caratteristiche morfologiche e instaurando le connessioni venose di quello destro; questo implica, tra le altre cose, lo sviluppo di due nodi seno atriali (NSA) e l’ingresso bilaterale delle vene cave destra e sinistra nei rispettivi atri. L’analisi morfologica è stata affiancata da un’estensiva analisi molecolare condotta prevalentemente mediante ibridazione in situ non radioattiva, allo scopo di valutare eventuali alterazioni del profilo trascrizionale del modello. La presenza di un secondo nodo ectopico e l’aver verificato un’alterata espressione di marcatori di sviluppo del sistema di conduzione ventricolare ci ha suggerito di investigare le proprietà elettrofisiologiche dei cuori embrionali. A tale scopo abbiamo adottato la metodica dell’optical mapping, conducendo uno studio in collaborazione con il Prof. David Sedmera (dell’Istituto di Fisiologa Animale e Genetica di Praga); la tecnica consente la registrazione dell’attivazione elettrica dell’epicardio del cuore con un’elevatissima risoluzione spaziale e temporale e la costruzione di mappe di attivazione del segnale che riproducono dove si generi e come si propaggia l’impulso stesso. Poiché i ventricoli non mostravano alterazioni drammatiche
della morfologia in assenza del gene, abbiamo infine effettuato un’analisi al confocale, allo scopo di evidenziare eventuali azioni cellulari del gene.

Lo studio qui presentato ha condotto alle seguenti conclusioni:

1 I topi cTP ko sono un buon modello di IAD giacché, in assenza del gene, la regione senoatriale destra si sviluppa secondo la morfologia della sinistra. L’IAD è uno dei tratti che rendono il fenotipo dei cuori cTP assimilabile a quello descritto in un ko costitutivo per Pitx2 caratterizzato in passato (Gage et al., 1999); l’IAD era invece completamente assente in un modello di ko condizionale generato più recentemente da Tessari et al. Tale modello differisce dal cTP per la cinetica di attivazione del promotore α-MHC che guida l’espressione della ricombinasi (Tessari et al., 2008): nei topi α-MHC, l’inattivazione di Pitx2 avviene con un giorno di ritardo rispetto ai cTP. Nel loro complesso, i nostri risultati indicano che l’espressione precoce di Pitx2 nelle cellule miocardiche, e non nei loro precursori mesenchimali, è una condizione necessaria e sufficiente per conferire identità “sinistra” alla regione senoatriale.

2 Pitx2 modula le proprietà trascrizionali del miocardio che circonda le vene cave e le vene polmonari: In assenza di Pitx2 si osserva la mancata repressione trascrizionale (nelle vene cave) o l’attivazione ectopica (nelle vene polmonari) di un programma genetico di tipo nodale, che comprende l’espressione di un canale del potassio, Hcn4, potenzialmente sufficiente a conferire autoritmicità ai tessuti (Stieber et al., 2003). In linea con tali osservazioni, l’analisi mediante optical mapping mostra che l’espressione del gene modula l’attività pacemaker del polo venoso del cuore in modo dose-dipendente. L’alterato profilo trascrizionale delle vene è un esempio di come l’attività di Pitx2 non si limiti alla morfogenesi (e quindi alla costruzione di strutture) ma si esplichi anche attraverso il conferimento di un’identità “sinistra” di tipo molecolare; la modulazione trascrizionale e l’azione morfogenetica possono essere svolte in modo concomitante e coordinato, oppure disgiunto e indipendente nel corso dello sviluppo. Le osservazioni condotte a livello delle vene dei mutanti cTP si inseriscono in un dibattito scientifico di acceso interesse circa il ruolo di Pitx2 nell’insorgenza della fibrillazione atriale (AF); alla luce di quanto osservato, la nostra opinione è che alterazioni dell’espressione di Pitx2 possano determinare l’insorgenza di attività pacemaker ectopica in vene cave e vene polmonari. La presenza di foci ectopici di attivazione elettrica rappresenta uno dei meccanismi meglio descritti nella patogenesi della fibrillazione atriale (Allessie et al., 2001; Nattel et al., 2002).

3 I cardiomiociti ventricolari ko per Pitx2 appaiono meno allungati e possiedono un minor grado di allineamento delle miofibrille rispetto ai wt; l’osservazione non è stata riprodotta in cardiomiociti atriali ko per il gene. Questo suggerisce un ruolo di Pitx2 nel processo di maturazione cellulare selettivamente svolto
nei ventricoli, dove potrebbe essere implicato nell’insorgenza delle alterazioni morfologiche riscontrate nei cuori cTP ko.

In conclusione, l’espressione miocardica di Pitx2 agisce in modo differenziale nelle diverse regioni cardiache: nella regione senoatriale conferisce identità morfologica, molecolare e funzionale, mentre nei ventricoli modula il rimodellamento delle camere.
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<td>a</td>
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<tr>
<td>Aa</td>
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<td>atrial fibrillation</td>
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<td>action potential or arterial pole</td>
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<td>ia</td>
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<td>PTA</td>
<td>persistent truncus arteriosus</td>
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<td>pt</td>
<td>pulmonary trunk</td>
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SHF  secondary heart field
som  somite
spm  splanchnic mesoderm
st   septum transversum
sv   sinus venosus
TGA  transposition of the great arteries
VSD  ventricular septal defect
v    ventricular region
vp   venous pole
vv   venous valves
1. INTRODUCTION

1.1 HEART DEVELOPMENT

The heart is the first organ to form during embryogenesis and because of its function of pump of the circulatory system is critical for embryonic viability and adult life; it is morphologically patterned along the three axes, including the left-right. Developmental abnormalities underlie many congenital cardiovascular malformations that represent the most common form of human birth defects. Although they are well characterized anatomically and physiologically, little is known on the genetic basis for most of these anomalies (Kitamura et al., 1999).

In mammals the heart has essentially a mesodermal origin, with a minor contribution of neural crest cells. Cells migrating from the primitive streak delaminate out and at E7.5 delineate a horseshoe shaped region in the splanchnic mesoderm underlying the head folds; this region is committed to the cardiogenic lineage during and immediately after gastrulation and is commonly called cardiogenic region or cardiac crescent, because of its characteristic shape (Figure 1).

At E7.75, in response to signals from the underlying endoderm, two endocardial tubes form laterally to the anterior gut endoderm; as the embryo undergoes lateral folding these two tubes are brought together toward the midline and fuse in an antero-posterior direction to form a single, primitive heart tube (E8.0). The cardiac crescent and the heart tube, derive from a lineage of cardiac precursors named first heart field (FHF); medially to the cardiac crescent and posteriorly to the primitive tube, a second distinct mesodermal lineage of progenitor cells lie, called secondary heart field (SHF) (Figure 1) (Buckingham et al., 2005).

As cardiac development proceeds, the earlier FHF-derived cardiac tube serves as a scaffold for the subsequent contribute of SHF-derived cells at its arterial and venous pole. The migration and integration of SHF-derived cells into the primitive heart tube allows its progressive growth and elongation, contributing almost exclusively to the development of the future arterial pole of the heart, the right ventricle, the atrioventricular canal and the pulmonary myocardium, whereas the FHF-derived portions of the tube uniquely contributes to the left ventricular myocardium and also to the atria (Buckingham et al., 2005). Thus, since the heart primordium forms, it is an embryologically heterogeneous organ where different cell populations with a distinct lineage and characterized by a specific gene expression pattern contribute to the formation of distinct myocardial regions.
Introduction

The primitive heart tube is composed of three layers: the innermost endothelium, an external myocardial layer, and a thick portion of acellular matrix secreted by the developing myocardium and deposited between them. The cardiac tube is oriented along the anterior-posterior axis, with venous tributaries draining into the developing atria at the posterior or inflow end, and the future arterial pole connecting to its anterior or outflow end.
Introduction

The heart tube is committed along the antero-posterior axis to form distinct prospective regions. Moving from the cranial to the caudal extremity of the tube, the following primitive structures will be delineating:

- the bulbus cordis
- the ventricular region
- the sinoatrial region

The primitive ventricular region will develop into the mature ventricular chambers. The superior end of bulbus cordis will become the truncus arteriosus which, after a process of septation, eventually splits into the helically arranged outflow regions of both ventricles: the ascending aorta and the pulmonary trunk. The truncus arteriosus is contiguous to an expansion named aortic sac that, in turn, connects with the aortic arches, the major arteries draining blood to the head and trunk. From the sinoatrial region the atrial chamber and the venous return will form; this last originally consist of the paired left and right sinus horns to which the common cardinal veins connect; they originate by the confluence of the posterior cardinal veins and the anterior cardinal veins, draining the trunk and the head region respectively. When the primitive tube starts to beat (E8) with a rhythmic peristaltic fashion, blood flows into a bilaterally symmetrical circulatory system: the paired cardinal veins drain the anterior and posterior sides of the body; then blood is pumped out into the right and left aortic arches.

At E8.25 the ventricular region of the heart tube bends rightwards (Figure 2); this asymmetrical event is highly conserved among all vertebrates. The dextral looping of the tube represents the earliest morphological marker for left-right patterning in vertebrate embryos and is followed by a coordinated folding of the tube in which

- the bulbus cordis is displaced inferiorly, anteriorly and to the embryo’s right
- the primitive ventricle moves to the embryo’s left side
- the primitive atrium and sinus venosus move superiorly and posteriorly (Figure 2).

Cardiac looping and the subsequent remodelling process are necessary to bring atria and ventricles in the correct reciprocal positions and to allow the alignment of the left and right ventricles with the: aorta and the pulmonary artery (or pulmonary trunk). At the end of the process (E10.5) the serially arranged cardiac segments has become arranged in parallel, thus allowing the heart to establish the normal ventriculoarterial connections. By mid fetal stages of development (E14.5 in mouse) the process is completed with chambers growth and septation, conduction system formation and valves development.
Introduction

Analysis of the role of the homeobox transcription factor Pitx2 in cardiac development: a conditional ko approach

After the establishment of the primitive tube, in addition to the splanchnic mesoderm of the first and second heart field, other three distinct lineages of cells contribute to the heart building:
- the sinus venosus myocardium
- the proepicardial organ (PEO)
- the cardiac neural crest cells (cNCC)

The sinus venosus myocardium progenitors originates from the caudal lateral part of the cardiac crescent separating and diversifying from the first and second heart fields very early in development (Mommersteeg et al., 2010); it will give origin to the sinoatrial node (SAN) and to the myocardial sleeves covering the systemic venous return (the future caval veins).
Introduction

The proepicardial organ is a mesenchymal transient structure localized anteriorly to the venous pole (Figure 1). Cells derived from the proepicardial organ migrate and cover the entire heart; these cells will form the visceral layer of the pericardium (i.e. the epicardium). Then, from E12.5, some of these epicardial cells undergo an epithelial-mesenchymal transition, thus becoming able to invade the myocardium where they differentiate into interstitial fibroblasts or into the smooth muscle cells of the coronary vasculature.

The cardiac neural crest cells have an ectodermal origin; they migrate from the dorsal neural tube across the posterior pharyngeal arches invading the outflow tract of the heart (Hutson and Kirby, 2007); in this region they contribute to the development of semilunar valves and are critical for outflow tract septation, that leads to the separation of the myocardial base of the pulmonary trunk and aorta.

Therefore the heart, that in its mature state works as a functional unit in a very elegant and coordinated fashion, from the embryological point of view is a modular, composite structure in which spatially and genetically distinct populations of cells are fated to contribute to distinct cardiac regions. Specific expression markers define these cardiac lineages and their transcriptional domains delineate the corresponding presumptive forming regions.

There is not a biunique correspondence between lineages and forming cardiac regions: more then a lineage can contribute to the development of a certain cardiac structure and, additionally, many distinct anatomical structures and cell types originate from every lineage, each of them driven by the activation of specific genetic pathways and signalling cascades.

Given the high level of complexity of cardiac development and morphogenesis a complete overview cannot be provided in this context and is outside the aim of this introduction; however extensive reviews on heart building have been published (Bruneau et al., 2002 and 2003; Moorman and Christoffels, 2003; Vincent and Buckingham, 2010). The basics about the crucial molecules and events involved in a definite developmental process will be reported when needed for the comprehension of the study.

1.2 PITX2

1.2.1 The Pitx gene family

The Pitx gene family is a bicoid-related family of homeobox-containing genes including three vertebrate paralogues. The first member of the family identified, the mouse Ptx1 (pituitary homeobox 1), was isolated as a transcription factor involved in pro-opiomelanocortin gene transcription in anterior pituitary gland (Lamonerie et al., 1996). Since the Ptx gene symbol had previously been assigned to some pentaxin genes in mouse and human, the official nomenclature of
Introduction

1.2.2 PITX2

The human PITX2 gene was identified by positional cloning of the 4q25 locus from a human craniofacial cDNA library and was found to be mutated in patients with Rieger syndrome (Semina et al., 1996). For this reason, it was originally called RIEG. Besides in human, Pitx2 genes have been cloned in mouse, chick, Xenopus and Drosophila.

In both human and mouse the PITX2 gene comprises six exons and expresses three major isoforms as a result of alternative pre-mRNA splicing (Pitx2a and Pitx2b) and alternative promoter usage (Pitx2c) (Gage and Camper, 1997; Kitamura et al., 1997) (Figure 3). The three isoforms contain identical C-termini, including the homeodomain spanning from exon 5 to a small portion of exon 6; on the
Introduction

contrary, they are substantially different in their amino-termini that hence may confer functional differences in *in vivo* contexts (Lamba et al., 2008). In 2002 a third mRNA isoform, Pitx2d, was cloned from a human craniofacial cDNA library (Cox et al., 2002). Pitx2d derives from the same promoter as Pitx2c, but is alternatively spliced at a cryptic 3’ splice acceptor site within exon 5, resulting in a truncated homeodomain that does not bind DNA but is able to inhibit the transcriptional activity of the other isoforms through direct physical interaction (Cox et al., 2002). The *Pitx2d* mRNA has been described only in human.

More recently, in both murine and human pituitary gland and skeletal muscle two additional Pitx2 isoforms have been identified, Pitx2cβ and Pitx2b2 (Lamba et al., 2008), produced via alternative translation initiation and pre-mRNA splicing. *In vitro*, these proteins show similar DNA binding and trans-activation properties as the other Pitx2 isoforms; however they could play distinct, although unidentified roles *in vivo* (Lamba et al., 2008).

**Figure 3:** The major murine Pitx2 isoforms

A: A map of the murine Pitx2 gene locus is represented. Exons (E) are numbered and indicated as boxes.; the homeobox, split by intron 5, is shown in black. B: Murine Pitx2 isoforms. Start (AUG) and stop (UGA) codons are marked by arrowheads; numbers indicate length of sequences (bp). From Schweickert et al., 2000.

Pitx2 proteins act as transcriptional regulators. Until now a limited number of genes have been demonstrated to be trans-activated by PITX2 in *in vitro* studies; these targets include procollagen lysyl hydroxylase 1 (*PLOD1*), atrial natriuretic factor (*Nppa*), prolactin (*PRL*), follicle-stimulating hormone β (*Fshb*), and luteinizing hormone β (*Lhb*) (Cox et al., 2002; Ganga et al., 2003; Suszko et al., 2003; Tremblay et al., 2000).

The three main PITX2 isoforms (-a, -b and -c) have both overlapping and distinct patterns of expression; Pitx2a and Pitx2b show symmetrical expression, whereas Pitx2c shows additional asymmetrical expression (see the paragraphe 1.2.5). Both the qualitative (localization) and the quantitative (gene dosage) features of their expression are critical for normal vertebrate development. In 1999 Gage et al. generated an hypomorphic (neo) and a null (-) allele for *Pitx2*,

- 7 -
obtaining an allelic series of Pitx2 mutations ranging from partial to complete loss of function. These mouse models allowed to show that multiple organs and distinct regions within the same organ differ in their sensitivity to Pitx2 deficiency, thus Pitx2 gene dosage requirement during organogenesis displays organ-, tissue- and regional-specificity (Gage et al., 1999).

### 1.2.3 PITX2 in disease

PITX2 is the causative gene for 4q25-linked cases of Rieger syndrome type I (RGS1, MIM 180500) (Semina et al., 1996). RIEG was first defined as a genetic disease by Rieger in 1935; the mutations reported in this disorder vary widely and include C-terminal truncations, point mutations in the homeobox helices and splice mutations (Kitamura et al., 1999). They mostly represent loss-of-function mutations, supporting that Rieger syndrome is caused by Pitx2 haplo-insufficiency; the correct gene dosage of Pitx2 is therefore essential to ensure its correct functionality in vivo. The main features of this autosomal dominant disorder are: structural malformations of the anterior chamber of the eye (cornea, trabecular meshwork and iris stroma) leading to glaucoma in more than 50% of those affected, dental defects (microdontia and anodontia), umbilical anomalies (ranging from protruding navel to omphalocele), and mild craniofacial dysmorphism (Rieger et al., 1935; Jorgensen et al., 1978). Occasionally, Rieger’s patients also present pituitary hormone deficiency and growth insufficiency, limb malformations and cardiac defects: aortic and pulmonary valvular stenosis, interatrial and interventricular septal defects, incomplete bundle branch block (Sadeghi-Nedjad and Senior, 1974; Tsai et al., 1994; Cunningham et al., 1998; Mammi et al., 1998).

A role for Pitx2 has been hypothesized in the etiogenesis of acute leukemia, since it was identified as a target of All1, the human homolog of Drosophila trithorax; in acute leukemias All1 is frequently subject to loss-of-function chromosomal translocations affecting Pitx2 expression as well (Arakawa et al., 1998). The precise action of Pitx2 in these tumors is still unknown.

Recently, independent genome-wide association studies have reported several risk variants on chromosome 4q25 that are strongly associated with atrial fibrillation in European and Asian populations (Gudbjartsson et al., 2007; Kääb et al., 2009). The SNPs map into a large intergenic region without any known gene (a so-called “genomic desert”); the closest gene mapping in the region is Pitx2. Subsequent in vivo studies in adult Pitx2 heterozygous mice (Wang et al., 2010; Kirchhof et al., 2011) have shown that Pitx2+/- hearts are more susceptible to AF during programmed stimulation than controls, and parallel microarray analyses have identified a wide range of Pitx2 transcriptionally affected left targets possibly mediating its action (Wang et al., 2010; Kirchhof et al., 2011; Chinchilla et al., 2011). Additionally, some of these last studies have detected and quantified Pitx2 expression in adult murine (Kirchhof et al., 2011) and human hearts (Chinchilla et al., 2011).
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providing for the first time evidences supporting a possible functional role of the gene also during adult life. Future studies will be required to identify the arrhythmic mechanism linking Pitx2 deregulation to AF pathophysiology.

1.2.4 The murine Pitx2 gene

The murine homologue of the PITX2 gene was cloned independently by several groups (Gage and Camper, 1997; Mucchielli et al., 1997; Kitamura et al., 1997; Lamba et al., 2008). Pitx2 has strong homology with the human sequence, sharing the 91% of nucleotide and the 99.2% of protein sequence identity; in particular, homeodomain is 100% conserved between mouse, human and chick also.

The developmental expression pattern of Pitx2 in mouse is consistent with the abnormalities reported in Rieger’s patients (Semina et al., 1996). The major sites of developmental expression are: the limbs and periocular mesenchyme (corresponding to the presumptive cornea, eyelids and extraocular muscles), the dental lamina, maxillary and mandibular epithelia, the gut, and the Rathke’s pouch (the precursor to the anterior and intermediate lobes of the pituitary gland). Pitx2 expression additionally patterns within tongue, kidneys, bone marrow, lungs, ventral mesoderm of the body wall and, early in development, in the left lateral plate mesoderm, in derivatives of the first branchial arch and within the brain (the developing prosencephalon and mesencephalon).

Additionally, Pitx2 is expressed in the developing heart: the earliest expression of the gene is on the left side of the late cardiac crescent at 2-4 somites (E7.8, Figure 4); at 6-8 somites (E8.0), when the primitive heart tube is formed Pitx2 transcripts are detected in the left prospective sinoatrial and ventricular region where Pitx2 continues to be expressed throughout looping (Figure 4). This morphogenetic process does not alter the placement of the atria with respect to the medial axes, therefore Pitx2 expression in the left portion of the venous pole and sinoatrial region becomes localized in the left atrial and venous region after looping completion (Figure 4). In contrast, as a result of cardiac morphogenetic movements, Pitx2 expression pattern in the developing ventricles has a very dynamic profile. Ventricular chambers, that were positioned along the embryonic anteroposterior axis, after looping become aligned with a left-right orientation, converting the original left-right differences in Pitx2 expression into dorso-ventral differences. As a consequence, the left-sided expression of Pitx2 in the primitive ventricular region and outflow tract becomes localized on the ventral surface of the left and right ventricles and in the left-ventral portion of the outflow tract as well (Figure 4).
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Therefore, as demonstrated by a fate map approach (Campione et al., 2001), the left regionalized expression of Pitx2 is maintained throughout cardiac morphogenesis and can be used as a bona fide lineage marker tracing the developmental fate of the left sided segments of the heart primordium.

1.2.5 Pitx2 in the left-right axis determination

The vertebrate body plan, whereas superficially bilaterally symmetric, contains an internal left-right (L-R) axis as revealed by the specific, non-random asymmetric arrangement of the organs in the chest.
and abdomen with respect to the midline. In the normal arrangement of organs, referred to as "situs solitus", the stomach, the spleen and the heart are located to the left of the body cavity, the liver is on the right side, intestines curl asymmetrically, and lungs display l-r differences in lobation. These asymmetries are evolutionarily conserved, underlining the importance of L-R differences in the development of the vertebrate body plan. Errors in L–R axis determination can lead to randomized and uncoupled morphogenesis (heterotaxia or "situs ambiguus"), complete inversion of the axis (called "situs inversus"), and mirror image duplications of organs (right or left isomerism: the condition where both sides of an organ assume the right- or left-side characteristics).

L-R axis is established with respect to the anterior-posterior (A-P) and dorsal-ventral (D-V) axes in a coordinated fashion to them. Current models divide the generation of left-right asymmetry into three steps: an initial breaking of embryonic symmetry, the establishment of an asymmetric signalling propagating along the embryo prior to any overt feature of L-R morphological asymmetry, and a last phase where individual organs interpret the cues that provide an instructive morphogenetic information and undergo asymmetric development (reviewed in Burdine et al., 2000; Hamada et al., 2002). This last phase can be mediated by three main morphogenetic processes: the directional looping of a tube (like in the primitive heart, spleen and digestive tube), the differential lobation of paired organs (as lungs), the asymmetric regression of initially paired structures (like blood vessels development).

Until now, no evidence supports that the upstream events leading to the initial break of the symmetry share a common mechanisms; on the contrary, vertebrates seem to have adopted very different, specie-specific strategies: the generation of the nodal flow in mouse, the presence of a L-R coordinator in frog, the unilateral transport of laterality determinants through intercellular gap junctions in chick and frog (Figure 5). Among the factors involved in the left asymmetrical signalling, a number of them have been shown to act only in one species and not in others, or having conflicting roles in different organisms. In contrast to these, transforming growth factor-β (TGF-β)-related molecules, have a conserved and pivotal role in all vertebrates and therefore represent the central components of the L-R pathway (Figure 5).
One of the earliest described as well as the most important of these strictly conserved asymmetric TGF-β signals is Nodal. Laterality of the internal organs of vertebrates is determined by the asymmetric and transient signalling of Nodal throughout the left lateral plate mesoderm (LPM), the mesoderm located in the lateral region of the early somite-stage embryo. The asymmetric expression pattern of Nodal is evolutionarily conserved in zebrafish, frog, chick, and mouse (Collignon et al., 1996; Levin et al., 1995; Lowe et al., 1996; Lustig et al., 1996; Rebagliati et al., 1998a; Sampath et al., 1998), and strictly correlates with visceral situs: deficiency of such signalling results in heterotaxia syndrome. Nodal signals are therefore determinants for “left-sidedness” in all vertebrates.

In all the model vertebrates examined, two additional members of the TGFβ family have been described to be crucial for the specification of L/R asymmetries: the Lefty proteins Lefty-1 and Lefty-2. These proteins lack a cysteine residue that is required for their dimerization, crucial to interact with their receptors. Genetic evidences indicate they act as antagonists of Nodal signalling: they are not left-side determinants per se, but work as regulators that restrict the spatio-temporal expression of Nodal in the LPM. Lefty-2 expression pattern in the left LPM mostly overlaps with that of Nodal.

Figure 5: Genetic cascades of L-R development in vertebrates
Genetic cascades of L-R development in mouse, chick, frog and Zebrafish. Green indicates conservation in at least two of the model organisms; blue indicates conservation in more than two of the model organisms; red indicates divergence among the model organisms. The dashed lines indicate the midline of the organism, with the left being to the reader’s left. For clarity, not all potential gene interactions are indicated. Note that TGFβ-related molecules have a conserved role in all vertebrates and Pitx2 induction in the left lateral plate mesoderm is the common response to the asymmetric signalling cascade. From Bardine et al., 2000.
and acts as a feedback inhibitor of Nodal signalling. *Lefty-1* is expressed on the left side of the embryonic floor plate, the most ventral part of the neural plate, and functions as a molecular midline barrier (Burdine et al., 2000).

The L-R signals emanating from the left lateral plate need then to be transmitted to the forming organs and translated into proper organogenesis, driving the cellular and molecular changes that underlie asymmetric morphogenesis at organ level.

Two opposing hypothesis were originally formulated about this point:

- each organ primordium responds to the left side-specific TGFβ signals by activating distinct target genes, which direct L-R asymmetric morphogenesis of that specific organ
- the left-sided factors activate the same genetic target in all the L-R asymmetric organ precursors of the embryo.

The transcription factor Pitx2 was identified as the candidate effector molecule interpreting left–right axial information from the early embryonic trunk to drive asymmetric morphogenesis in each organ (Lu et al., 1999). The reasons supporting the hypothesis that Pitx2 could execute such a function are the followings:

1) Pitx2 is induced by Nodal asymmetric signalling throughout the left side of the lateral plate mesoderm; this step of L-R asymmetric development is widely conserved in vertebrates and has been independently demonstrated by different groups in mouse, chick, frog (*Xenopus*) and fish (*Zebrafish*) (Ryan et al., 1998; Yoshioka et al., 1998; Logan et al., 1998; Piedra et al., 1998; Campione et al., 1999; Cheng et al., 2000).

2) Compared to that of Nodal, Pitx2 expression domain is larger: it includes not only the left lateral plate mesoderm but also the left side of organ primordia that, subsequently, develop asymmetrically (the left lung bud, the left heart primordium, the left portion of the forming gut).

3) Nodal is only transiently active in the left LPM and terminates before organogenesis takes place.

Pitx2 asymmetric expression is initiated by Nodal signaling; after down-regulation of TGF-β signals, Pitx2 expression is maintained by the homeobox transcription factor Nkx2 interacting with a specific Nkx2 binding site on a Pitx2 enhancer (Shiratori et al., 2001). This TGF-β-independent induction of transcription allows Pitx2 to persists in the developing handed organs as they undergo L-R-specific morphogenesis. These observations strongly supported that Pitx2 induction in the developing organs was the common response to the asymmetric signalling cascade, able to direct the morphogenetic events underpinning the L-R specificity of organs in the thorax and abdomen. Additionally, both gain- and loss-of-function experiments in different animal models have validated this hypothesis.

In chicken, ectopic application of Shh on the right side of Hensen’s node causes ectopic expression of Nodal on the right side and misexpression of Pitx2 in the right LPM of the embryo; this is sufficient to
induce randomization of heart looping, body rotation, and gut situs as well as aberrant organ morphogenesis (Levin et al., 1995; Isaac et al., 1997; Logan et al., 1998; Ryan et al., 1998) and demonstrating a role for Pitx2 downstream to Nodal in the gene cascade that controls laterality of heart and gut. Pitx2 expression pattern is perturbed in mouse models with laterality defects; in these mutants, aberrations in Pitx2 expression overlap in terms of pattern and frequencies with the altered expression of Nodal, consistently with the genetic frame in which Nodal works as the inducer of Pitx2. In turn, Pitx2 misexpression correlates with heart and gut situs inversion and/or aberrant morphology, suggesting that Pitx2 paricipated in the late phase of the genetic cascade controlling organ positioning and morphogenesis. (Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998).

Pitx2c but not Pitx2a or Pitx2b is asymmetrically expressed in the left LPM and in developing heart, lungs and gastro-intestinal tract; Pitx2c is also specifically induced by Nodal (Schweickert et al., 1999). Therefore, Pitx2c represents the left-specific isoform acting as the downstream mediator of the left-right pathway for imparting and maintaining “leftness” during vertebrate asymmetrical development. In 2001 Liu et al. have generated two Pitx2 alleles encoding progressively higher levels of Pitx2c in the absence of the other isoforms, demonstrating that different organs require distinct levels of Pitx2c activity for their correct asymmetrical patterning. The different sensitivity to Pitx2c gene dosage in organ asymmetric development can be due to different mechanisms: in each organ Pitx2c may bind targets with different affinity binding sites in their regulatory elements; alternatively, Pitx2c could be differentially regulated at transcriptional or post-translational level and/or tissue-specifically interact with proteins modulating its transcriptional activity. Answering this point will allow to understand how the same gene (Pitx2) induced in a variety of organ precursors by the left-side-specific TGFβ signals can lead to distinct responses in each different context.

1.2.6 Pitx2 in cardiac morphogenesis

Mice with complete Pitx2 loss-of-function alleles die by embryonic day (E) 14.5 due to severe heart defects compromising haemodynamics and circulatory system functionality (Lu et al., 1999). The pleiotropic expression of the gene determinates a complex phenotype with multiorgan abnormalities and severe external features that make easily recognizable the Pitx2−/− mutants. Normal E8.5 embryos start turning along the A-P axis in a clockwise direction leading to a change of the embryo curvature, that moves from a lordotic curvature to a dorsal kyphotic bend of the spinal column (Theiler et al., 1989; Farlex et al., 2008). Pitx2−/− embryos show a correctly initiated counterclockwise rotation; however turning is arrested, placing the mutant lower trunk to the right of the upper trunk. Pitx2-deficient mice also exhibit failure of ventral body-wall closure, a patent umbilical ring and evisceration of abdominal content, with heart and abdominal organs externalized.
In Pitx2\(-/-\) embryos, the earliest event of asymmetric cardiac morphogenesis is spared and heart tube undergoes normal, rightwards looping (E8.5); however, most of the later events of cardiac morphogenesis and remodelling are disrupted leading to a very complex cardiac phenotype. The main features of Pitx2-deficient hearts are the right sino-atrial isomerism (RAI) and a severe ventricular dismorphogenesis. The RAI is a pathological condition in which the asymmetric left-right patterning of the sinoatrial region is lost and both the atrial chambers develop the anatomical features and the venous connections of the right one. In Pitx2\(-/-\) hearts the atrial appendages develop as a single, common atrium having a symmetrical morphology, bilateral sinoatrial nodes and venous valves, and a symmetrical arrangement of the sinus horns (the prospective caval veins). In addition, the failed looping results in a rightward, dorsal displacement of the atroventricular canal, the outflow tract and the mutant ventricles, these last lying on the same dorso-ventral plane as the outflow tract. Also the remodelling movements are arrested, leading to abnormal maintenance of the serial arrangement of cardiac chambers (typical of the primitive heart tube) and to failure in the establishment of the normal ventriculoarterial connections. This misalignment results in a double outlet right ventricle (DORV, where both of the great arteries connect to the right ventricle ) or in a transposition of the great arteries (TGA, in which the pulmonary artery and the aorta are reversed and connect to the wrong ventricle). A diminished growth of the right ventricular myocardium has been additionally described, resulting in a right ventricle hypoplasia (Kioussi et al., 2002; Kitamura et al., 1999).

Other morphological alterations include interatrial and interventricular septal defects and an atroventricular canal with prominent swelling; the heart fails to develop tricuspid and mitral valves and a common atroventricular valve forms with hyperplasia of the cushion tissues. In Pitx2\(-/-\) mice cardiac orientation is also clearly altered and hearts develop with the apex incorrectly directed rightward instead than leftward (Lu et al., 1999).

The complex Pitx2\(-/-\) phenotype includes a set of severe extracardiac abnormalities, in line with the major sites of expression of the gene (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999; Liu et al., 2001;). In particular, Pitx2\(-/-\) mice exhibit craniofacial malformations (with major dental and ocular defects), failure in pituitary gland development, splenic hypoplasia and displaced, right isomeric lungs (Lu et al., 1999).

Pitx2 is therefore essential for many aspects of normal development, with pivotal roles in eye, pituitary gland, teeth, and heart. Nevertheless, the complexity of the Pitx2 constitutive knock-out phenotype makes very difficult to dissect the cardiac action of the gene, as the severe anomalies found in the Pitx2\(-/-\) mice might be secondary to organs evisceration, or/and to the contribution of extracardiac Pitx2-positive cells from the LPM or/and to a general arrest of the developmental
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processes caused by the absence of this essential gene. Hemodynamics has been demonstrated to exert a crucial role in the regulation of a number of morphogenetic processes (Poelmann et al., 2008; Sankova et al., 2010), therefore presumptive alterations in hemodynamics of Pitx2\(^{-/-}\) hearts can also contribute to determine the mutant phenotype. In line with this, Yashiro et al. have described a non-cell-autonomous role of Pitx2 in the situs-specific, asymmetric development of the aortic arch. The formation of the aortic arch takes origin from the asymmetrical remodeling of a primitive symmetrical arterial system, in order to develop the mature arrangement where the aortic arch is placed on the left side. A crucial event of this remodelling is the complete regression of the right sixth branchial arch artery (BAA) and the right dorsal aorta, since the final situs of the aortic arch depends on which side the sixth BAA is maintained. Pitx2 promotes BAA remodelling in a non-cell-autonomous manner: it drives the rotation of the outflow tract, thus leading to the generation of an asymmetric haemodynamic flow in the paired sixth BAA. As a result, a differential activation of the PFGFR and VEGFR2 signalling occurs leading to the asymmetric development of the aortic arch. The molecular mechanism through which Pitx2 drives the rotation of the arterial pole has not been investigated (Yashiro et al., 2007).

The exact role of Pitx2 in cardiac morphogenesis is not known. In order to selectively focus on its cardiac action it is appropriate to analyse Pitx2-null mutants that lack asymmetric expression of Pitx2 specifically in the cardiac muscle. In line with this, in 2008 Tessari et al. have investigated the Pitx2 myocardial function in a conditional ko mouse model generated by crossing Pitx2 floxed mice with α-myosin heavy chain deletor mice, having efficient myocardial Cre activity from E8.5. The ko mice survived to adulthood and presented abnormalities in the ventricular remodelling; however, the resulting morphological and functional defects did not recapitulate the features described in the Pitx2\(^{-/-}\) mice and displayed a reduced penetrance and a variable expressivity; additionally, the severe sinoatrial phenotype found in the constitutive Pitx2 ko was not present at all (Tessari et al., 2008).

Thereby, a good murine model to investigate the role of the gene during heart development is still missing so far. For this reason we have generated a novel myocardial conditional Pitx2 ko mice where the action of the gene is prevented from early cardiogenesis; the characterization of the model is the object of this study.
2. AIM OF THE WORK

Cardiac morphogenesis is an extremely critical process for the correct functionality of the cardiovascular system: errors in the morphogenetic program cause many structural and functional abnormalities in the heart and the great vessels, named as Congenital Heart Disease (CHD). A CHD is present in approximately 1% of newborns and is the direct cause of the 20% of miscarriages and of perinatal mortality in the 10% of live births (Hoffman and Christianson, 1978; Hoffman et al., 1995).

Although they are well characterized anatomically and physiologically, little is still known about the genetic basis for these anomalies but in most of the congenital cardiovascular malformations developmental abnormalities underlie their onset (Benson et al., 1998; Kitamura et al., 1999); therefore, to investigate the role of the genes directing cardiac development is an essential step to understand how the pathological conditions arise.

Pitx2 is an homeodomain transcription factor expressed during embryogenesis and crucial for the development of many organs i.e. eyes, teethes, pituitary gland and heart (Gage et al., 1999). Additionally, Pitx2 acts as the downstream mediator of the left-right pathway for imparting and maintaining “leftness” during the morphogenesis of the asymmetrical organs (Levin et al., 1995; Isaac et al., 1997; Logan et al., 1998; Ryan et al., 1998). Both these actions are exerted in the heart, which is asymmetrically patterned and where the gene is expressed from organ primordium formation and throughout all cardiac development (Campione et al., 2001). Nevertheless, how Pitx2 carries out its morphogenetic action is unknown so far.

In order to analyze its function in vivo, murine models lacking Pitx2 expression have been generated. The Pitx2-null mice die during the mid-fetal stage of development; their characterization (Lu et al., 1999) has shown a complex set of severe cardiac and extra-cardiac abnormalities, including the failure of body wall closure with evisceration of the abdominal content; however, the complexity of the phenotype makes difficult to dissect the cardiac action of the gene, since the major abnormalities described in the Pitx2-null hearts might be secondary to the severe systemic effects produced by the loss of the gene. More recently, a myocardial-specific ko has been generated and analyzed (Tessari et al., 2008). The ko mice survived to adulthood and did not recapitulate the severe cardiac phenotype described in the Pitx2−/− mice: milder morphological and functional defects were found in ventricular chambers, with a reduced penetrance and a variable expressivity; moreover, the complex sinoatrial phenotype found in the constitutive Pitx2 ko was not present.

Thereby, in order to dissect the myocardial action of Pitx2 in heart development, we have generated a novel Pitx2 conditional ko in which the expression of the gene is prevented from early cardiogenesis, including in cardiogenic precursors. The morphological, molecular and functional characterization of the model is the object of this work.
Aim of the work

Analysis of the role of the homeobox transcription factor Pitx2 in cardiac development: a conditional ko approach
3. MATERIALS AND METHODS

3.1 MOUSE LINES

3.1.1 The cTnT Cre strain
The cTnT Cre mice were provided from Kai Jiao (Howard Hughes Medical Institute). These mice have been obtained by injection into the pronuclei of one cell embryos of a vector containing the nld-Cre-hGH cassette fused with the rat cTnT promoter (Wang et al., 1994). In the resulting transgenic line, transcription of Cre recombinase is under control of the rat cardiac Troponin T (cTnT) promoter (Jiao et al. 2003).

3.1.2 The R26R reporter strain
The in vivo monitoring of Cre-mediated excision events and the analysis of the Cre recombinase efficiency has been tracked using the R26R mouse strain. The R26R allele was produced by targeted insertion in the mouse ROSA26 locus of a DNA fragment containing a splice acceptor sequence, a neo expression cassette flanked by loxP sites, a lacZ gene and a polyadenylation sequence. A triple polyadenylation sequence to the 3’ end of the neo expression cassette was added to prevent transcriptional read-through (Soriano et al. 1999). Interbreeding R26R mice with the cTnt Cre transgenic line, the neo cassette is excised by Cre recombinase activity and lacZ expression is conditionally restored in the cTnT- positive cells.

3.1.3 The Pitx2 Floxed strain
The murine Pitx2 Floxed strain was obtained in 2001 from Philip Gage (University of Michigan). In the Pitx2flox allele the DNA binding homeodomain exon 5 of the gene is flanked with the LoxP sequences; Cre-mediated recombination and excision of this exon, produces a null allele due to the loss of the critical homeodomain and a frameshift leading to missense codons and a premature stop codon (Gage et al. 1999).

3.2 EMBRYOS COLLECTION AND STORAGE
Mice were mated and every morning females were checked for vaginal plug formation (9-10 A.M.); day 0 of gestation is the day the vaginal plug was found. The embryos collected on each gestational day for our analysis were heterogeneous pools collected from many females. Stage E8.5, E10.5, E14.5, E17.5 embryos were isolated and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C, washed in PBS, dehydrated through a series of ethanol/PBS solutions (25%, 50%, 75% ethanol), and stored at −20°C until use.
3.3 SAMPLES CUTTING

Embryos were dehydrate through a graded ethanol series (80%, 90%, 100% ethanol), embedded in paraffin and microtome sectioned at 12 µm. The sections were put on a film of water on Superfrost Plus(+) Poly-L-Lysine-coated slides (Thermo Scientific) and heated to 40°C to allow section stretch; then water was removed and sections were dried at 40°C for at least 30 min on a heated plate.

3.4 MOUSE LINES GENOTYPING

DNA for PCR screening was extracted with illustra tissue & cells genomicPrep Mini Spin Kit (GE Healthcare) from newborn and adult tails of anaesthetized mice (Zoletil, 30mg/kg, i.p). Amniotic sac and limb pieces of embryos isolated after sacrifice of the anaesthetized mother, were lysed in the Viagen DirectPCR® DNA Extraction System (Viagen); the resulting DNA extracts are compatible with genomic PCR for genotyping. For PCR screening of the Pitx2 floxed allele and to assess the presence of the Cre and Rosa26R sequences, the following set of primers were used:

PCR Pitx2

Pitx2 forward 5’-tcg tgt ctt aaa agg atg tgt ttc tt-3’
Pitx2 reverse 5’-ttc tgg agg gtt ttc ttg ttc tag g-3’

The expected band weight were 1200bp for the floxed allele, 950bp for the wt allele, and 500bp for the deleted allele.

PCR Cre

Cre forward 5’-cgt ttt ctg agc at acct gga-3’
Cre reverse 5’-att ctc cca ccg tca gta cg-3’

The expected band size was 500bp.

PCR Rosa

Rosa forward 5’-gga gcg gga gaa atg gat atg-3’
Rosa reverse1 5’-aaa gtc gct ctg agt tgt tat-3’
Rosa reverse2 5’-gag aag agt tgt tcc tca acc-3’

Cycling conditions for all the three amplification reactions were:

4 min at 95°C
34 cycles of 30 sec at 95°C
30 sec at 60°C
1 min and 30 sec at 72°C
10 min at 72°C for the final extension step.
3.5 X-GAL HISTOCHEMICAL STAINING

Transgenic mouse embryos were isolated and rinsed for 2-3 times in Phosphate-buffered saline (PBS) 1X to eliminate the blood. Samples were briefly fixed in freshly 4% paraformaldehyde (PFA) at room temperature (RT) for a time depending on the embryonic developmental stage and the tissue dimension. Embryos were rinsed again in PBS 1X to eliminate surplus of PFA and incubated in X-gal solution at RT for 2 hours at 37°C. The composition of X-gal solution is the following; final concentrations are indicated:

0.5 mg/ml X-gal
0.01% Triton X-100 (Sigma)
Magnesium chloride 2 mM
Potassium ferricyanide 5 mM
Potassium ferrocyanide 5 mM
Phosphate-buffered saline 1%

After rinsing embryos, a 2 hours fixation in 4% PFA has been performed for a better and long term preservation of the staining.

3.6 HEMATOXYLIN AND EOSIN (H&E) STAINING

Sections were deparaffined by washing in Xylene (3 times for 10 min), dehydrated through a graded ethanol series and equilibrated with phosphate buffered saline buffer (PBS). Hematoxylin and Eosin Staining was performed using the Rapid Frozen Sections H&E staining kit (Bio-Optica) according to the manufacturer’s specifications and mounted in Entellan new (Merck).

3.7 RIBOPROBES SYNTHESIS

Complementary RNA probes to full length mouse Pitx2 (Campione M et al., 1999), Tbx3 (Hoogaars et al., 2004), Shox2 (Espinoza-Lewis et al., 2009), Hcn4 (Stieber et al., 2003), Tbx18 (Kraus et al., 2001), Nkx2.5 (Lyons I et al., 2009), Cx40 (Hoogaars et al., 2004), Nppa (Zeller R et al., 2011), BMP10 (Chen H et al., 2004), and the ATP-binding site of myosin heavy chain (MHC) (Boheler et al., 1992) were generated. Plasmids containing the DNA sequences encoding for the antisense RNAs were a generous gift of the authors quoted above, except for the Cre and ID2 riboprobes that have been cloned in our laboratory. Plasmids were linearized by restriction enzyme digestion. DIG-labeled, single stranded RNA probes were synthesized by in vitro transcription using an SP6, T7 or T3 RNA polymerase. The labeling assay has been performed in RNase free water using the DIG RNA Labeling Mix (Roche) and a Protector RNase Inhibitor (Roche) according to the manufacturer’s specifications. RNA probes were then purified from labeling reactions with mini Quick Spin Columns (Roche).
3.8 NONRADIOACTIVE IN SITU HYBRIDIZATION

3.8.1 Nonradioactive whole mount in situ hybridization

Keeping embryos on ice, they were rehydrated through ethanol series and washed three times for 5 min in phosphate-buffered saline (PBS) plus 0.1% Tween® 20 (PBT). Embryos were pretreated by a proteolytic digestion with proteinase K (10 μg/ml in PBT) at room temperature for a time depending on the embryonic developmental stage and sample dimension. The proteolytic digestion was blocked washing embryos for 5 min in 2 mg/ml glycine in PBT; they were then rinsed in PBT and washed twice in PBT for 5 min each. Samples were re-fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBT for 15 min at room temperature, rinsed in PBT and washed three times in PBT for 5 min each. Samples were subsequently washed in 50% hybridization mix/ PBT, followed by a washing in 100% hybridization mix. Replacing 900 µl of fresh hybridization mix in plastic vials, embryos were pre-hybridized at 65°C for at least 1 hour. Embryos were then incubated overnight at 70°C in a water bath, after adding 100 µl of hybridization solution containing 2 µl of denatured riboprobe stock.

On the second day, embryos were rinsed in 50% formamide/5x SSC, pH 4.5 /0.1% Tween-20 and then washed twice for 30 min in the same solution. Embryos were then washed twice in 50% formamide/2x SSC, pH 4.5 for 30 min each at 70°C. Embryos were then rinsed three times for 5 min in PBT, blocked for 2 h at room temperature in B-block by rocking, and incubated overnight at 4°C in B-block with 1:3000 sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) by rocking.

On the third day, embryos were washed in PBT three times for 10 min and in NTM twice for 10 min each. The staining reaction was performed overnight in BM Purple Agent substrate precipitating (Roche), keeping samples in the dark. Stained embryos were fixed overnight in 4% PFA, washed in PBT and photographed under a Leica stereomicroscope. For long-term storage, samples were dehydrated through ethanol series and kept in ethanol 75% at -20°C.

Composition of solutions

Hybridization mix: 50% formamide, 5 x SSC, 1% Blocking Reagent (Roche), 5 mM EDTA, 0.1% Tween-20, 0.1% Chaps (Sigma), 0.1 mg/ml heparin (Sigma), and 1 mg/ml baker’s yeast tRNA (Roche).

B-block: 1% Blocking Reagent (Roche), 10% goatserum in PBS-T.

NTM: 100 mM NaCl, 100 mM TrisHCl pH 9.5, and 50 mM MgCl₂.
3.8.2 Nonradioactive in situ hybridization on sections

Sections were deparaffined by washing in Xylene (3 times for 10 min), dehydrated through a graded ethanol series and equilibrated with phosphate buffered saline buffer (PBS). Sections were then pretreated by a proteolytic digestion for 5–15 min at 37°C with 20 μg/ml proteinase K dissolved in PBS for a time depending on the embryonic developmental stage and the tissue dimension. Protease permeabilization was followed by a 5-min rinse in 0.2%glycine/PBS and two rinses of 5 min in PBS. Sections were then re-fixed for 20 min in 4% paraformaldehyde/0.2% glutaraldehyde dissolved in PBS to ensure firm attachment of the sections to the microscope slides, and washed twice in PBS for 5 min. Samples were pre-hybridized in hybridization mix without probes for 1 hr at 70°C in a humid chamber immersed into a water bath; the humid chamber was saturated with 50% formamide/ distilled water. Keeping the same condition of the pre-hybridization step, sections were then hybridized overnight with the digoxigenin labelled riboprobes diluted in the hybridization mix. The optimal concentration for each riboprobe was calibrated; the volumes applied to the sections spanned between 2-8 μl depending on the embryonic developmental stage and section size. Before adding the probes, they were denatured by heating at 95°C for 5 min.

After hybridization, sections were rinsed in 2× SSC pH 4.5, and washed three times for 30 min at 65°C in 50% formamide/2 × SSC, pH 4.5. Followed three 5-min washes in PBS plus 0.1% Tween® 20 (PBT), and 1 hr of pre-incubation in B-block. Probe bound to the section was immunologically detected using sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) diluted 1:2000 in B-block. Sections were washed three times for 5 min in PBT, followed by two 5-min washes in NTM. Subsequently, overnight incubation in the chromogenic substrate NBT/BCIP Stock Solution (Roche) 1: 50 in NTM was performed. Slides were rinsed with double-distilled water, dehydrated in a graded ethanol series and xylene, mounted with Entellan and photographed under a Leica DMR microscope using a DC300 digital camera.

Composition of solutions

Hybridization mix: 50% formamide, 5 × SSC, 1% Blocking Reagent (Roche), 5 mM EDTA, 0.1% Tween-20, 0.1% Chaps (Sigma), 0.1 mg/ml heparin (Sigma), and 1 mg/ml baker’s yeast tRNA (Roche).

B-block: 1% Blocking Reagent (Roche), 10% goatserum in PBS-T.

NTM: 100 mM NaCl, 100 mM TrisHCl pH 9.5, and 50 mM MgCl₂.

3.9 MRI ANALYSIS

Magnetic resonance imaging of wt and mutants E14.5 embryos, was performed by Shoumo Bhattacharya’s laboratory on a horizontal 9.4T/21 cm VNMRS Direct Drive MR system (Varian Inc., Palo Alto, CA, USA).
3.10 OPTICAL MAPPING

E14.5 embryos, obtained from matings of Pitx2loxP/loxP and cTnT Cre Pitx2loxP/wt mice, were dissected on ice, their heart isolated and pieces of limbs kept for subsequent genotyping. Hearts were stained for 10 minutes with the voltage sensitive dye di-4-ANEPPS (Invitrogen) at 4°C.

Then they were pinned, their dorsal part up, on the bottom of a silicone-lined copper dish positioned on a temperature-controlled stage (TH 60, 40°C) of an upright epifluorescence microscope (Leica DML-FS). The copper dish was filled with Tyrode-HEPES solution (pH 7.4) oxygenated and additioned with Blebbistatin, in order to uncouple cardiac excitation from contraction, thus limiting motion artifacts.

For consistency, and because of complex atrial geometry obscuring the pacemaker site, all recordings were performed in dorsal view, with the posterior atrial wall facing the optical apparatus. To accommodate the entire heart, x 4 and x 10 water dipping objectives and 0.63 photo tubes were used, resulting in individual pixel size between 16-40 μm.

Experiments were performed on a setup based upon Olympus BX51WI fixed stage upright microscope equipped with a wide green band-pass (480-550 nm) excitation and red (590 nm LP) emission filter set, so that drops in fluorescence intensity corresponded to passing of action potentials.

Images were recorded for 2 and 4 sec at a rate of 2048 frames/s with a high-speed Ultima L camera (SciMedia, Japan) and recordings were processed digitally using BV_Analyzer software (SciMedia, Japan). The peak of numerically computed first derivative (dF/dt max) exceeding set threshold was considered the time of activation for the respective pixel. Isochronal activation maps were generated with a 3x3 spatial filter in a 16 or 32 ms temporal window and classified with respect to pacemaker location and direction of impulse propagation.

3.10.1 Criteria for samples exclusion

Samples not beating or not supported by optimal data captures were excluded from our analysis. In order to avoid experimental biases and artefacts that could affect the soundness of the classifications, we additionally excluded those samples, within all genotypes, showing abnormally prolonged atrial activation times (t > 13ms) and/or altered atrial propagation patterns. In particular a cranial line of block was observed among all genotypes (n= 18), as previously described by Abramochkin et al (2009). This region seems to be physiologically more sensitive to experimental variables (medium temperature and oxygenation, muscle fatigue, dye phototoxicity) and prone to slow down impulse conduction.

Additional samples (n=9) were excluded for the presence of an interatrial conduction block, characterized by normal conduction speed in each atrium but extended total time of atrial activation; this abnormality can be explained by damage at level of Bachmann’s bundle, possibly as a consequence of sample dissection, resulting in a break of the interatrial impulse transmission.
In some additional samples (n=12) of all genotypes, the activation maps showed an atrial impulse originating at multiple sites and a beat to beat shift of pacemaker location. This pacemaker shift was often associated with the presence of a cranial (n=5) or a bundle branch block (n=3) but in some samples (n=4) it has been observed in absence of any other alteration of the atrial activation pattern.

### 3.10.2 Statistical analysis

The atrial activation and propagation patterns observed in our wt and mutant hearts could be grouped into three categories, each having a minimum of 10 hearts. Differences between groups were analyzed using Pearson's $\chi^2$-test; values of $P < 0.05$ were considered statistically significant. Atrial activation times were calculated as an average between 4 × and 10 × recordings of each sample when these numbers did not differ more than 2 ms. Data are presented as mean ± standard deviations; comparisons between wt, cTP het and cTP ko values were performed using a two-tailed Student’s t-test; $P$-values < 0.05 were considered significant.

### 3.11 IMMUNOFLUORESCENCE AND CONFOCAL ANALYSIS

Staged E14.5 wt and cTP ko embryos were fixed for 1 hour in 4% paraformaldehyde (PFA) equilibrated overnight in sucrose 30% in PBS and freezed in liquid nitrogen. Embryos were sectioned using cryostat and immunostained with monoclonal anti actinin (1:200, Capra) antibody; actinin localization was revealed by anti-mouse-TRITC conjugated secondary antibody (1:200 Dako). Nuclei were counterstained using Hoechst 33342 dye. Imaging system consisted of a Leica inverted microscope DM IRE2 and a Leica true confocal scanner TCS SP2 equipped with Ar/ArKr, He/Ne543/594 and He/Ne 633 lasers. Images were recorded using a Leica PL APOx63/1.4 oil immersion objective after performing 10 μm Z-scan, with 1 μm. step.
4. RESULTS

4.1 CHARACTERIZATION OF THE cTnT Cre Pitx2 (cTP) MOUSE LINE

We have investigated the myocardial role of Pitx2 with a conditional ko approach by crossing Pitx2 floxed mice (Pitx2\textsuperscript{loxP/loxP}) (Gage et al., 1999) with TroponinT (TnT) Cre deleter mice, which display efficient myocardial Cre activity from early cardiomyogenesis (Jiao et al., 2003). Indeed, TnT Cre mRNA was visible from E7.5 in the cardiac crescent (Figure 6A,a), whereas Cre activity, assessed by X-Gal histochemical staining, was detected in the same region at late presomitic stage (Figure 6A,b) and by E8.5 was visible in the whole heart (Figure 6A,c). In this line, a Pitx2 PCR on genomic DNA from trunk (t) and isolated heart (h) of a E8.5 cTP ko embryo (Figure 6A,d) shows the cardiac-specific recombination of the Pitx2 locus and the amplification of the excised fragment in the heart and not in the rest of the body. Hence, in our mice the recombinase activity is detectable one day earlier than in the previously characterized conditional ko model (Tessari et al., 2008; see section 1.2.6), thus preventing even the earliest myocardial Pitx2 expression. The LoxP sequences flank the exon 5, therefore the Cre recombinase activity promotes the genomic excision of a major portion of the DNA binding homeodomain, making the protein functionally inactive. In this Cre-LoxP model all the three isoforms of the protein are silenced (-a, -b, and -c); however, since the Pitx2c is the only isoform detectable in the heart, the phenotype displayed from our mice is attributable to the loss of this specific isoform.

Selective myocardial deletion of one loxP allele (TnT Cre; Pitx2\textsuperscript{loxP/wt} = Pitx2 het\textsubscript{myo}, from here referred as cTP het) resulted in viable and fertile offspring; no obvious morphological defects were visible in cTP hets (not shown). Additional animals were subsequently generated by crossing cTP hets with Pitx2\textsuperscript{loxP/loxP} mice. By genotype analysis cTP ko were found dead in the cages at postnatal day (P)3; since genotype distribution at late stages of development (E17.5) was according to the Mendelian ratio (not shown) we concluded that the cTP ko phenotype is perinatally lethal.
Results

Figure 6: cTP mouse line characterization
A: TnT Cre line characterization. Onset of Cre mRNA expression visualized by whole mount ISH (Cre) (a) and onset of Cre activity (b), assessed by crossing TnTCre with R26R mice (LacZ); c: by E8.5 Cre activity is visible in the entire heart; d: Pitx2 PCR on genomic DNA from trunk (t) and isolated heart (h) of a E8.5 cTP ko embryo to assess cardiac-specific recombination at the Pitx2 locus. M1: Lambda phage DNA, BstEII digested; M2: 100bp ladder. From the embryonic trunk only the floxed allele is amplified (1232 bp band). In the corresponding heart only the 500 bp band is visible, indicating complete Cre-driven recombination. B-C: H/E staining and MRI analysis of E14.5 wt and cTP ko hearts. e: arrow indicates the ASD, dotted circle indicates abnormal shape of the left ativoventricular valve; f: arrowhead indicates the VSD; red stars show symmetrical pectinate muscles arrangement in atria. C: h,i: ventriculoc-arterial alignment defects in cTP ko and (h’-i’) their corresponding 3D MRI reconstructions. ao: aorta; asd: atrial septal defect; cc: cardiac crescent; hf: headfolds; ias: interatrial septum; ivc: inferior caval vein; ivs: interventricular septum; la, ra: left and right atrium; lscv, rs: left and right superior caval veins; lv, rv: left and right ventricle; pa: pulmonary artery; pv: pulmonary veins; san: sinoatrial node; tr: trachea; vsd: ventricular septal defect; vv: venous valves. Scale bar: 0.5mm

Analysis of the role of the homeobox transcription factor Pitx2 in cardiac development: a conditional ko approach
Table 1: Characterization of cTP cardiac defects by MRI analysis

<table>
<thead>
<tr>
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<th>wt(n=7)</th>
<th>het(n=5)</th>
<th>ko(n=5)</th>
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<td>0</td>
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</tr>
<tr>
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<tr>
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<tr>
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<td>0</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>left</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>1</td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<td>7</td>
<td>5</td>
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<tr>
<td>into common atrium</td>
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<td>5</td>
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<tr>
<td>PV drainage</td>
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<td>from LV</td>
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<tr>
<td>from RV</td>
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<td>PA exit</td>
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</tr>
<tr>
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<td>4</td>
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<tr>
<td>DORV</td>
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</table>

Histological and MRI analysis of E14.5 mutant hearts revealed a complex phenotype (Figure 6B-C; Table 1). In the cTP ko the left auricle was identical in shape and orientation to the right one (Figure 6B,d-f) and the atrial septum was reduced or totally absent (atrial septal defect, ASD) (Figure 6B,e). In a normal heart the sinoatrial node (SAN) is a right-sided structure, located at the junction of the superior caval vein with the right atrial chamber; moreover, in a normal venous return the caval veins drain the blood from the systemic...
circulation to the right atrium and the pulmonary veins connect to the left atrium draining the oxygenated blood of the pulmonary circulation. In the cTP ko hearts we detected bilateral SANs, bilateral caval veins (CV) entrance into the atria, and drainage of the inferior caval vein (ICV) and pulmonary vein (PV) into the medial part of the common atrium (Figure 6B,d-f). Overall, these are typical features of right atrial isomerism (RAI), a pathological condition where both the atrial chambers develop the anatomical features and the venous connections of the right one. Therefore, the early myocardial Pitx2 action is necessary and sufficient to confer left identity to the sinoatrial region and the cTP ko mice are a good model of RAI. Additionally, we detected a ventricular septal defects (VSD) and an abnormal atrioventricular junction (AVJ) displaying a swelling of the left atrioventricular (AV) valve (Figure 6B,e) and. Moreover, ventriculoarterial alignment was abnormal in the cTP ko, resulting in transposition of the great arteries (TGA, where the pulmonary artery and the aorta are reversed and connect to the wrong ventricle) and double outlet right ventricle (DORV, in which both of the great arteries connect to the right ventricle) (Figure 6C and Table 1). Altogether, the myocardial cTP ko recapitulated the major phenotypic features of Pitx2 null mice, thus indicating that Pitx2 morphogenetic action is required from early cardiogenesis.

4.2 THE cTP SINOATRIAL REGION

4.2.1 Preliminary remarks: the sinus venosus myocardium

In human and mouse embryos the sinus venosus (SV) myocardium is a myocardial region including the sinoatrial node (SAN) and the sinus horns (SH), from which the myocardial walls of the right and left superior caval veins (RSCV, LSCV) will be differentiating (Christoffels et al., 2006; Sizarov et al., 2010). The SV myocardium is originated by a single genetic cell lineage of Nkx2.5 negative mesenchymal precursors expressing the T-box Tbx18; these mesenchymal cells are originally located (E8.25 in mouse) at the lateral rims of the splanchnic mesoderm (Mommersteeg et al., 2007). Among them, SAN precursors can be bilaterally identified at the most lateral borders of the Tbx18+ mesenchymal domain, in a small subdomain coexpressing the LIM homeodomain transcription factor Isl1 (Cai et al., 2003; Mommersteeg et al., 2010) whereas the myocardial SAN primordium will be recognizable from E9.5 onward at the junction between the right atrium and the right superior caval vein.

The SH can first be identified at E9.5 as a small myocardial region medially located with respect to the systemic veins entrance into the common atria. By E10.5 caval veins entrance shifts towards right and concomitantly the SH starts to expand (Christoffels et al., 2006; Sizarov et al., 2010, Soufan et al., 2004), progressively delineating the myocardial sleeves covering caval veins (CV) (Christoffels et al., 2006).

In adult hearts, the systemic venous return and the myocardium covering it are asymmetrically arranged: the right SH is mostly incorporated into the right atrium (RA), whereas the left superior caval vein (LSCV) drains into the RA through the coronary sinus (CS) in mouse and the brachiocephalic vein in man.
The molecular mechanisms driving the progressive reorganization from bilateral SV mesenchymal precursors into a lateralized SAN and an asymmetrically structured SH myocardium have not been clarified so far.

4.2.1.2 Preliminary remarks: cardiac patterning into working and primary myocardium

In line of principle all the myocardial cells are autorhythmic and therefore able to generate an electrical impulse; this is functionally true in the myocardium of the primitive heart tube, which is initially composed of slowly conducting myocytes all possessing intrinsic pacemaker activity. On the other hand, the adult heart has a specialized electrical conduction system, mainly formed by the sinoatrial and atrioventricular nodes (SAN and AVN), the His (or atrioventricular) bundle and the bundle branches (Figure 16A). The development of these specialized structures leads to the coordinated and sequential contraction of atrial and ventricular chambers, in contrast with the peristaltic contractions of the primitive heart tube.

The cells of the conduction system are functionally different from the cells forming the atrial and ventricular chambers because of their being more prone to automaticity than to contraction. At the cellular level, this functional property requires a more pronounced tendency to depolarize spontaneously and poor coupling between cardiomyocytes, which are achieved by a peculiar composition and density of ion channels and gap junctions as well as by the cellular size and arrangement. Frequent, spontaneous depolarizations and poor intercellular coupling allow cells to load until a threshold value resulting in the generation of an action potential triggering myocardial contraction.

On the contrary, an effective ejection of blood from cardiac chambers requires a synchronous contraction of myocardial cells, that are this time very efficiently coupled in order to quickly propagate the depolarizing impulse. These chamber cardiomyocytes display no apparent automaticity and additionally have well-developed sarcomeres and sarcoplasmic reticular structures conferring them a high contractility. Atrial and ventricular myocardial cells have these functional properties and are altogether named working myocardium in opposition to the non-contracting myocardium (including conduction system components) that is defined primary myocardium. His bundle and bundle branches have an intermediate behaviour between the “primary” and the “working” profile, since they have a prominent pacemaker activity (like the SAN and the AVN) and also a high conduction velocity (like the atrial and ventricular cardiomyocytes). In absence of unambiguous morphological criteria to distinguish the different populations of cells, this functional classification is normally used to follow and recognize the different regions of the developing heart (Figure 7).
The opposite functional features of the working and primary myocardium correspond to different transcriptional profiles activated with regional specificity. In 2003 Moorman and Christoffels described this fine expression pattern regulation with a model based on the genetic interplay between the homeobox protein Nkx2.5 and two T-box factors: Tbx5 and Tbx2. Nkx2.5 is one of the master transcriptional regulators for cardiogenesis, from early cardiac commitment of mesodermal cells (it is one of the first markers for cardiac crescent) to septation and chambers formation at later stages. Nkx2.5 can physically interact with both Tbx5 and Tbx2. The interaction with Tbx5 leads to the formation of a transcriptional complex activating a “chamber-type” genetic program. This results in the expression of specific marker genes of the working myocardium: the connexins CX40 and CX43 and the Nppa gene, encoding for the atrial natriuretic factor (ANF), a peptide hormone involved in regulation of blood pressure during adult life. On the contrary, Tbx2 has an expression pattern complementary to Tbx5 and delineating the primary myocardium: the inflow and outflow tracts, the inner curvature and the atrioventricular canal (i.e. the region interposed between the developing chambers). In presence of Nkx2.5, Tbx2 can repress the localized differentiation into working myocardium by repressing Nppa, CX40 and CX43 genes (Habets et al., 2001). Therefore, the transcriptional regulation of target genes is performed by Nkx2.5 and T-box transcription factors through interaction with NK factor binding elements (NKE) and T-box bindings elements (TBE) on their promoters; the switch between the positive or negative transcriptional control, and the regional specificity of the action is driven by Tbx5 in the working and Tbx2 in the primary myocardium (Figure 8).
4.2.1.2a Molecular specification of the sinoatrial node (SAN)

In subdomains of the primary myocardium, the additional interaction with the T-box Tbx3 will drive the development of all the main components of cardiac conduction system (CS). The role of this T-box transcriptional repressor has been well characterized in the sinoatrial node (SAN). Tbx3 activation in the SAN and not in the rest of the sinus venous-derived myocardium is critical to suppress the activation of a working myocardium genetic program (including the genes encoding for the connexins CX40 and CX43, the inwardly rectifying potassium channels Kir2.1, Kir2.2, Kir3.1, the sodium channel Nav1.5 and the atrial natriuretic factor, ANF); concomitantly, genes of the “nodal” genetic profile are indirectly induced (the calcium channel gene Cav3.1, the connexin Cx30.2 gene, the potassium channels genes Kv1.1, Hcn1, Hcn2 and Hcn4), resulting in a unique gap junctions and ion channels composition that confers to the SAN its pacemaker function (Hoogaars et al., 2007; Mommersteeg et al., 2007). In this line, one of the most important event in the development of the nodal tissue is the induction of Hcn4, encoding an isoform of a voltage-gated potassium channel that strongly contributes to the “funny” current (I\textsubscript{f}) required for the spontaneous activity of the pacemaker cells (Stieber et al., 2003). Hcn4 is highly expressed in human and mice sinoatrial and atrioventricular nodes and is critical for their autorhythmic properties (Stieber et al., 2003). Additionally, before the formation of any nodal structure, Hcn4 expression in the developing heart tube in strictly associated to the location of the dominant pacemaker activity (Mommersteeg et al., 2007); for these reasons Hcn4 is commonly used as a specific functional marker for pacemaker tissues (Stieber et al., 2003; Milanesi et al., 2006).
The loss of \( Tbx3 \) expression in the SAN leads to the expansion of the atrial genetic program within the node (Hoogaars et al., 2007). On the other hand, the ectopic expression of \( Tbx3 \) in atria causes the transcriptional repression of genes encoding for *working myocardium* proteins and the overexpression of pacemaking genes, including \( Hcn4 \); these molecular changes overall result in arrhythmias and ectopic atrial pacemaking (Hoogaars et al., 2007).

### 4.2.1.2 Molecular maturation of the caval veins: shift toward a working genetic program

The SV plays a crucial functional role during development: at early stages this region presents autorhythmic properties and works as the leading pacemaker site of the primitive tube (Van Mierop et al., 1967; Kamino et al., 1981; Sedmera et al., 2006; Vicente-Steijn et al., 2010). When caval veins and SAN develop, they run the same genetic program and share the same functional, authorythmic properties, at least until mid-fetal stages (E14.5), when they begin to diverge in term of molecular pattern and cellular features. Indeed, it has been described that from E14.5 onward the caval veins myocardium, but not the SAN, progressively matures to obtain a *working*-type molecular profile comparable to that of the atrial myocardium, with up-regulation of fast conducting connexins and down-regulation of the pacemaker channel \( Hcn4 \) (Mommersteeg et al., 2007). The functional implication of this process is that pacemaker activity (that during the early embryogenesis was a sinus venosus-wide property) becomes progressively confined to a small subdomain of the sinus venosus-derived myocardium: the SAN.

### 4.2.1.3 Preliminary remarks: formation of the pulmonary myocardium

As the systemic return, the pulmonary veins are normally covered by a myocardial layer of cells with a still unclear function. The myocardial sheath coating the pulmonary venous return is known as the pulmonary myocardium and it was originally thought to form from migration of atrial myocardial cells around the pulmonary veins; this notion was based in part on the observation that pulmonary myocardium and atria run a common a *working myocardium* genetic program. The hypothesis was definitively refuted in 2007 when, using a lineage tracing approach, Mommersteeg et al. described a biphasic process for pulmonary myocardial sleeves formation. In the first step (E11.5-12.5), a myocardial cell population forms at the junction between veins and left atrium by *de novo* differentiation of \( Pitx2 \)-expressing mesenchymal cells with a SHF origin; a second phase of rapid proliferation follows from E12.5 onwards, allowing these \( Pitx2 \)-positive myocardial cells to coat the veins for their entire length.

In \( Pitx2^{+/−} \) mice, the pulmonary myocardium is absent since the initial pool of differentiated pulmonary cardiomyocytes fails to form. Apoptosis was not found in the pulmonary mesenchyme of these mice, therefore authors conclude that \( Pitx2 \) is not required to maintain these cardiomyocytes cells, but for
directly inducing their differentiation or, alternatively, for juxtaposing pulmonary mesenchyme with a presumptive unknown inducing tissue.

4.2.2.1 Results: formation of the ectopic sinoatrial node (SAN)

ISH analysis showed that in cTP ko embryos both SANs were myocardialized and correctly expressed the nodal differentiation and functional markers Tbx18, Tbx3, Shox2 and Hcn4, but not the working myocardium markers Nkx2.5 and CX40 (Figure 9); this prompted us to investigate the role of Pitx2 in the earliest events of asymmetric right-SAN formation.

SAN cardiomyocytes constitute a subpopulation of the SV myocardium, which is derived from Tbx18 expressing mesenchymal precursors. At E8.5 Tbx18 positive SV progenitors are symmetrically located at the lateral rims of the splanchnic mesoderm (Mommersteeg et al., 2010) (Figure 10A, arrowheads), flanking the proepicardial organ (peo). At the most lateral borders of this region, SAN precursors can be identified by their coexpression with the secondary heart field marker Isl1 (Mommersteeg et al., 2010); the left portion of the SV mesenchyme, including the left SAN precursors, additionally co-expresses Pitx2 (Figure 10A). In E10.5 wt embryos, the embryonic SAN structure is right-sided and can be identified as a thickening of Isl1 and Tbx18 positive myocardial cells at the border between the RSCV and the RA (Mommersteeg et al., 2007) (Figure 10B). The corresponding region on the left side is not myocardialized (Figure 10B). On the contrary, in E10.5 cTP ko embryos, the borders between the CVs and atria were both myocardialized and co-expressing Isl1 and Tbx18, thus indicating the presence of a second left-SAN (Figure 10B), which presented a correct molecular pattern (not shown).

Since our cTP ko model is myocardial-specific, the presence of an ectopic myocardial structure in the cTP ko embryos could seem an apparent paradox. Our interpretation is that Pitx2 normally prevents the expansion of the left Isl1/Tbx18-positive SAN mesenchymal precursor cells at the onset of their differentiation into cardiomyocytes. In the cTP ko mice, where Pitx2 action is loss, cardiomyocytes of the SAN primordium can expand bilaterally and originate paired SANs.
Figure 9: The left SAN of the cTP ko presents correct molecular pattern
ISH analysis of E14.5 wt and cTP ko embryos to assess left-SAN molecular signature. Note SAN expression of MHC, Hcn4, Tbx3, Shox2, Tbx18, and the negative staining with Nkx2.5 and CX40 antisense probes. Scale bar: 0.2mm
Results

Figure 10: Left SAN mesenchymal precursors differentiate and expand into the left ectopic SAN of the cTP ko
A: ISH of E8.5 wt embryos shows Tbx18 expression in SV mesenchymal precursors (arrowheads) and proepicardial organ (peo); Isl1 is bilaterally expressed in the second heart field (shf) overlapping Tbx18 at the SAN progenitors (dotted squares; green: right; red, left). Pitx2 expression delineates the left SV domain (arrowheads), including left SAN progenitors (green dotted square). B: At E10.5, cTP ko embryos present bilateral MHC, Isl1, and Tbx18 positive regions at the borders between left and right cardiac veins (lcv, rcv) and atria, identifying the early differentiated bilateral SAN. Scale bar: 0.1 mm
Results

4.2.2.2 Results: morphological and molecular characterization of the caval veins

Pitx2 expression in the entire left-SV mesenchymal region (Figure 11A) prompted us to analyze its possible additional role in the left SV-derived caval vein (CV) myocardium. As described in the previous section (----), caval veins originate from the sinus horn (SH); the first SH cardiomyocytes can be identified at E9.5, medially located with respect to the entrance of the systemic veins into the common atria (Christoffels et al., 2006). By E10.5 this symmetry is lost since the systemic veins entrance progressively shifts towards right; concomitantly, at the lower wall of the left superior caval vein (LSCV) (Christoffels et al., 2006), a myocardial structure named as the coronary sinus (CS) is formed in mouse, draining blood from LSCV to the right atrium (Sizarov et al., 2010; Soufan et al., 2004).

In E14.5 wt embryos, ICV entrance is positioned on the right (Figure 6B,a and dotted circles in Figure 11A,b), corresponding to the area of RSCV confluence with the CS. On the contrary, in the cTP ko ICV enter medially into the common atria (Figure 6B,d and dotted circles in Figure 11A,c), the CS is absent and LSCV and RSCV run symmetrically. This can be outlined by ISH with the hyperpolarization-activated channel Hcn4, which at this stage presents a SV-wide expression (Figure 11A,b). Given the myocardial-specific loss of Pitx2 in our model, we conclude that symmetric organization of the CVs is due to the lack of a Pitx2-dependent left cardiomyocyte expansion into the CS structure, which is missing in the cTP ko; as a consequence of the failed CS myocardium expansion, the entrance of the systemic veins into atrial chamber is not displaced on the right and an immature, medial position is maintained.

The systemic venous return is covered by a myocardial layer of cells originating from the SV myocardium that form by E12.5 from Tbx18-positive Nkx2.5-negative mesenchymal precursors and progressively expand until covering all the CVs (Christoffels et al., 2006). In E14.5 wt embryos, LSCV myocardialization is restricted to its most proximal portion; these SV cardiomyocytes express Pitx2 (Figure 11B,a-d). At E17.5 the LSCV myocardium extended more distally (Figure 11B,e-f), however this was not observed in the cTP ko (Figure 11B,g). We therefore concluded that loss of Pitx2 affect caval veins cardiomyocyte expansion both at early stages (during coronary sinus formation) and at late developmental stages.

We then tested if Pitx2 could modulate the LSCV transcriptional properties. As described before (section 4.2.1.2b), a progressive shift towards an atrial-type gene expression program is started in the CV myocardium at E14.5 and is strongly visible by E17.5 (Mommersteeg et al., 2007) (Figure 11C): Nkx2.5 is upregulated in both the SAN and CVs, whereas the gap junction Cx40 is upregulated exclusively in CVs, without obvious left-right differences; concomitantly, Hcn4 expression is downregulated exclusively in the LSCV (red arrows in Figure 11C,e). Hcn4 is responsible for generation of pacemaker potentials (Stieber et al., 2003), therefore at late fetal stages the RSCV myocardium retains a more nodal-type phenotype than the LSCV.
This atrial-type gene program normally detectable in developing caval veins was affected in the cTP ko hearts, where it was restricted to the most proximal, myocardialized region (red bars in Figure 11C,b,d,f) and it was activated in a incomplete fashion: Nkx2.5 and Cx40 expression were upregulated as in the wt.
samples, but Hcn4 expression was not repressed (Figure 11C,f). As a result, the nodal-type molecular profile was bilaterally retained.

4.2.2.3 Results: molecular characterization of the pulmonary myocardium

Pitx2 is normally expressed on the left side of the SHF-derived dorsal mesocardium from which pulmonary veins develop (Figure 12A); this suggests that pulmonary veins are patterned along the left-right axes and indeed they normally connect to the left atrium draining the oxygenated blood from the pulmonary circulation. This left positional information is lost in the cTP ko hearts where pulmonary veins open medially into the common atrial chamber, as shown in previous sections (Figure 6B,d).

As described above, pulmonary veins are covered by a myocardial layer named pulmonary myocardium, and Pitx2 is critical in the differentiation of the Pitx2-positive mesenchymal precursors into cardiomyocytes of the pulmonary myocardium (Mommersteeg et al., 2007; section 4.2.1.3) which retains Pitx2 expression (Figure 12B).

Figure 12: Pitx2 delineates the left side of the dorsal mesocardium and the deriving pulmonary myocardium, where it is crucial for its distal extension.
A: ISH on sections of E10.5 wt hearts showing Pitx2 expression on the left side of the MHC-negative dorsal mesocardium (in the dotted, red rectangle); the black line represents the midline. B: ISH of E14.5 wt hearts; the pulmonary myocardium covering the pulmonary veins (black arrows and black boxes) expresses Pitx2. C: ISH analysis showing myosin heavy chain expression in the pulmonary veins (black rectangles) of wt and cTP ko hearts at E14.5 and E17.5; red bars indicate the border of probe detection. In wt hearts the pulmonary myocardium extends for the entire length of the veins, whereas in the cTP ko samples it is confined to their most proximal part; a: common atrial chamber; la,ra: left and right atrium; lv,rv: left and right ventricle; pv: pulmonary veins; st: septum transversum; v: common ventricular chamber.
Results

Pitx2-null mice lack this myocardial region; analysing the wt and cTP ko pulmonary myocardium we found that the myocardialization of pulmonary veins was impaired and/or delayed in the cTP ko (Figure 12C). This suggests that Pitx2 is important not only in the first step of pulmonary myocardium differentiation (as described by Mommersteeg et al.), but it continues to exert an active role even in myocardial cells, sustaining the rapid proliferation that allow them to coat pulmonary veins for their entire length.

By in situ hybridization on section, we have then characterized the expression pattern of normal pulmonary vein myocardium at middle (E14.5) and late stages (E17.5) of gestation (Figure 13). Nkx2.5 is one of the markers of the SHF (the lineage from which pulmonary myocardium originates), and pulmonary veins retain its expression during all the development. The inhibitor of DNA binding 2 (ID2) and the short stature homeobox 2 (Shox2) are respectively molecular markers delineating the ventricular conduction system and SAN development; we found they are also expressed in the pulmonary veins with a temporally dynamic pattern: from E14.5 to E17.5, the first is strongly repressed and the latter is modulated with a distal-proximal gradient. Moreover, at both stages pulmonary veins express the conducting gap-junctional protein CX40 and do not express the Tbx3 and Hcn4 messengers, in line with its working genetic profile (Figure 13). Therefore, in normal embryos, from its formation till late development, pulmonary myocardium has a molecular profile that can be assimilated to the atrial one, since it runs a CX40-positive, Tbx3-Hcn4-negative gene program.

Figure 13: Pulmonary myocardium molecular profile
ISH analysis of E14.5 wt pulmonary veins (in the black rectangles) to assess the molecular signature of the pulmonary myocardium. Note the expression of CX40, Nkx2.5, Shox2, ID2 and the negative staining with the Tbx3 and HCN4 antisense probes.
In absence of Pitx2, Nkx2.5 and CX40 expression was unaffected, whereas the late modulation of ID2 and Shox2 was lost (Figure 14); moreover, the ectopic expression of the Tbx3 and Hcn4 genes has been detected, revealing an identity shift of the cTP ko pulmonary myocardium toward a nodal-like genetic profile (Figure 14).

Concluding, loss of Pitx2 expression in the myocardium of cTP mice affects the correct laterality of pulmonary veins entrance into the atrial chambers, which becomes medial instead of left-sided; cTP ko hearts present an incomplete myocardialization of the pulmonary veins, possibly due to an impaired proliferation and/or migration of these cardiomyocytes; in the absence of Pitx2 the pulmonary myocardium molecular properties are affected: a nodal-like molecular identity is acquired.

4.2.2.4 Results: electrophysiological characterization of the sinoatrial region

Given the presence of a left ectopic SAN in the cTP ko mice, we decided to investigate the functional properties of mid-fetal mouse hearts (E14.5) in order to understand if the left node, correctly patterned (Figure 9), was functional.

We decided to approach this problem by optical mapping technique; this tool provides epicardial recordings of the cardiac electrical activation with high temporal and spatial resolution, giving an ex vivo physiological readout about patterns and times of impulse generation and propagation. Recordings were performed in dorsal view and spatiotemporal activation maps were classified with respect to pacemaker location and direction of impulse propagation (Figure 15). Details on data processing and analysis and criteria for samples inclusion/exclusion are presented in the Materials and methods (section 3.10).

The functional properties of mid-fetal mouse hearts are quite elusive so far, therefore we needed to characterize the features of the atrial electrical activation in wt at first. In most of the wt hearts (n=36) the
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site of first breakthrough was detected in the RA (n=29; 80%) mainly around the R-SAN region, in a smaller group (n=6; 17%) it mapped medially, in correspondence of the caval veins, while a single wt sample presented a left-sided origin of the impulse (n=1; 3%) (Figure 15A). The direction of action potential (AP) propagation was mostly sequential from RA to LA (n=28; 78%), but synchronous bilateral propagation in the two atria was also recorded (difference ≈ 1-2 ms in n=8; 22%) (Figure 15B). Therefore, the mid fetal stage sinoatrial region has a certain degree of functional plasticity, as both the SAN and caval veins exhibit pacemaker activity and are able to originate the electrical impulse. Both the site of the first atrial breakthrough and AP direction of propagation presented a variable distribution within the sinus venosus (SV) region, with a pronounced right-sided dominance.

In the cTP ko hearts (n=10), where a left-SAN is present, the distribution of activation and propagation patterns was different. We found a reduced percentage of samples having a right-sided (n=6; 60%) or mid-dorsal (n=1; 10%) impulse initiation, and a higher incidence of left originating breakthrough (n=3; 30%) (Figure 15A). Bilateral AP propagation was not detected, whereas a new left-to-right direction of impulse spread was significantly present (n=4; 40%). In one additional ko sample a double concomitant impulse firing was observed, with the two earliest activated sites located in the R- and L-SAN regions (Figure 15C).

The concomitant presence of a left- and a right-sided origin of impulse, never found in any other wt or cTP het sample analyzed, lead us to infer that this unique activation pattern is due to the activity of the two SANs caught in the act to generate the electrical impulse almost simultaneously. We therefore conclude that the L-SAN is functional. The cTP het samples (n=17) appear morphologically normal; in particular they do not have the ectopic L-SAN. Nevertheless, they presented an intermediate behaviour both in pacemaker location distribution and in the impulse propagation profile (Figure 15A and B).

In conclusion, our optical mapping analyses have revealed a certain degree of functional plasticity in E14.5 hearts, which display pacemaker activity at both the R-SAN and CVs. In wt hearts this SV-wide capacity to generate the first electrical activity is mainly restricted to the right side, where the SAN is located; when Pitx2 gene dosage is reduced (cTP het) or its action is lacking (cTP ko), a left pacemaker potential is progressively uncovered (Figure 15B). Therefore, Pitx2 prevents the occurrence of a left pacemaker activity in the SV myocardium in a dose dependent way, thus restricting impulse generation to its right side, where the SAN is located.

Moreover, correlation between pacemaker location and AP spread direction (Figure 15D) highlights that, given a site of impulse initiation, atrial propagation patterns are different in the three genotypes, indicating differences in their conduction properties. In line with this observation, we found that time of atrial impulse propagation (Figure 15E) is significantly higher in cTP ko hearts compared to wt and het (wt= 7,3ms; het= 7,3ms; ko= 9,0ms).
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Figure 15: Ptx2 represses left pacemaker activity within the SV myocardium in E14.5 embryos
A: Site of first breakthrough. Top: prototypical maps (dorsal view) showing the three main atrial activation patterns recorded in isolated E14.5 hearts by optical mapping. The earliest activated region is indicated with an asterisk. Isochronal lines, delimiting regions activated within the same time frame, are spaced at 1 ms intervals; colour progression visualizes the advancing activation wavefront. Below: diagram showing the distribution of the activation patterns in the three genotypes. n= classified samples. B: Direction of impulse propagation. Top: three representative examples of observed impulse propagation patterns; arrows depict the direction of electrical activation spread. Below: diagram illustrating pattern distribution within the genotypes. P values < 0.05 are indicated. C: Activation map of a cTP ko heart showing the almost synchronous activation of the two SANs. D: Correlation between site of first atrial breakthrough and direction of impulse propagation. Columns indicate the combined distribution of atrial activation and propagation patterns within genotypes. Data are expressed as percentages; N.O. = never observed. E: Atrial activation times presented as averages ± standard deviations; asterisks indicate P < 0.05.
4.3 THE cTP VENTRICULAR REGION

4.3.1 Molecular characterization

As described in section 4.1 the first analysis about the morphological alterations of the cTP model has revealed an interventricular septal defect and abnormalities in the ventriculo-arterial alignment (transposition of the great arteries and double outlet right ventricle) (Fig 6Ch and h’, i and i’); these anomalies are often secondary effects of a ventricular dismorphogenesis so they can be considered per se a ventricular phenotype of the cTP ko mice.

In order to understand if they were associated with an altered molecular pattern, we performed an extensive characterization of ventricular tissue by in situ hybridization whole mount and on section. First we investigated the expression of genes encoding for transcription factors (i.e. Irx4, dHand and eHand) or other proteins (i.e. BMP10 and ANF) normally expressed during ventricular chambers development, but none noticeable alteration have been found (not shown). Then we have analyzed the ventricular developmental genes expressed with a regional specificity, focusing in particular on markers expressed at level of the interventricular septum, where the only morphological alteration had been observed. In this line we analysed the patterning of two important components of the ventricular conduction system: the His bundle, which is located on the top of the IVS, and the left and right bundle branches extending laterally to it (Figure 16A). The cells of these structures have a poorly developed contractile apparatus and t-tubular system, but they are very well-coupled expressing at high level the connexins CX40 and CX43 in order to rapidly conduct the impulse; additionally, His bundle and bundle branches express a common developing marker: the T-box gene Tbx3.

We therefore performed in situ hybridizations on four-chamber sections of E14.5 normal and mutant hearts using probes against the CX40 and Tbx3 markers which coexpression allows to easily identify and visualize the forming atrioventricular bundle and its branches. At this stage of development, the Tbx3 signalling clearly define the His bundle on the top of the interventricular septum (Figure 16B) and the bundle branches laterally to it; these last in addition express CX40 (Figure 16B). In the corresponding regions of cTP ko hearts, the signal for Tbx3 is reduced (in the AV bundle) or absent (in the bundle branches) (Figure 16B); CX40 expression is also reduced and confined to a thin monolayer of cells compared to the control (Figure 16B). Hence in mice lacking myocardial expression of Pitx2 we found a topological alteration in the expression of developmental (Tbx3) and functional (Cx40) markers for AV bundle and bundle branches.
3.2 Functional characterization

Since the only noticeable molecular deregulation reported in the cTP ventricles was affecting components of the ventricular conduction system, we wondered whether these molecular modifications were associated to a functional impairment of their electrical activation. We approached this problem...
performing an optical mapping analysis on ventricles of isolated E14.5 hearts; all the recordings were obtained in dorsal view.

Ventricular recordings correspond to the epicardial projections of bundle branches activity; their electrical activation leads to the formation of two waves of activation, on the left and on the right of the interventricular septum which is represented in the figure with a dotted line (Figure 16C). Once originated from the branches, the electrical impulses spreads to the rest of the ventricular camera.

The picture on the left of the Figure 16 shows a representative optical map of a wild type sample where the left bundle branch is activating before the right one; this is the most common pattern found in the wt condition (n=43/52; 83%), probably due to the anatomical peculiarity of the left bundle that, soon after its emergence from the fibrous septum, fans out broadly toward the posterior surface of ventricles. With a minor frequency, the almost synchronous activation of both the branches have been observed whereas only occasionally was the right bundle that activated first. No differences in the type of the activation patterns and their distribution have been found both in the cTP het and in the cTP ko samples (Figure 16); therefore, the ventricular electrical activation of mutants is normal, with both the bundle branches being functional and correctly propagating the impulse to the ventricular chambers. Total ventricular activation times were also evaluated and results were comparable within the three groups of samples (wt= 4.7ms; het= 4.5ms; ko= 5.0ms). Therefore, the topological alteration of Tbx3 and CX40 transcriptional domains observed by in situ hybridization did not lead to a functional impairment as assessed by optical mapping.

4.3.3 Cellular aspects

Since in our cTP model we did not observe any macroscopic alteration in terms of ventricular morphology and function, we decided to perform a confocal analysis in order to highlight possible more subtle alterations caused by Pitx2 absence, at cellular level. We focused in particular on cellular maturation.

Embryonic and mature cardiomyocytes are very different cells. Embryonic myocardial cells have a polyhedral shape with myofibrils running randomly in the sparse cytoplasm and cell-cell contacts distributed along the entire sarcolemm; in contrast, mature late-fetal cardiomyocytes are characterized by an extremely well-organized cytoarchitecture, achieved by multiple steps (Hirschy et al., 2006). During fetal development cardiomyocytes undergo an oriented elongation correlated with myofibrils lengthening and their parallel alignment, whereas the polarized restriction of the intercalated discs to the ends of the myofibers is a much slower process, that continues and is completed only postnatally (Hirschy et al., 2006).

Since our cTP ko model is perinatally lethal we investigated only the earliest events of cardiac developmental maturation performing an immunofluorescence staining of the Z-line protein α-actinin, outlining the contractile apparatus. The stainings were performed taking into account the peculiarity of Pitx2 ventricular transcriptional domain that, as a consequence of the heart looping movements, becomes
Results

Analysis of the role of the homeobox transcription factor Pitx2 in cardiac development: a conditional ko approach

patterned along the dorso-ventral instead of the original left-right axis (see the section 1.2.4 in the introduction).

In a E14.5 heart, Pitx2 is expressed on the ventral surface of the ventricles and not on their dorsal side (Figure 17), but this expression is not homogenous. Indeed the in situ hybridization on a four-chamber section shows that at E14.5 Pitx2 transcripts localize in the left atrium, in the interatrial septum, on the right side of the growing interventricular septum, in the compact myocardium of the ventral base of the right ventricle and all around the site where aorta comes out from the left ventricle (the so-called retroaortic ring bundle, RARB) (Figure 17); these transcriptional domains have been represented in a 3D model where the Pitx2-positive regions are in blue and the Pitx2-negative are in white (Figure 18).

First of all we compared the wt and ko myocardial cells from corresponding Pitx2-positive regions: the right side and the base and of the interventricular septum. In wt ventricles, Pitx2-positive cardiomyocytes present an elongated shape and a clear myofibrillar alignment, as revealed by sarcomeric α-actinin stripes, whereas in Pitx2 ko cardiomyocytes from the same corresponding region α-actinin have a spotted signal and lack organization, nuclei are rounded and there is no sign of cellular alignment and elongation (Figure 18A,B). The same staining was then performed on ventricular regions normally not expressing Pitx2 i.e. the right ventral wall, where we obtained a comparable result in the wt and in the cTP ko samples. In these Pitx2-negative cells, the myofibrillar alignment was well visible but less pronounced compared to the region expressing the gene; additionally, myofibers were less elongated and had rounded nuclei (Figure 18C). The analysis of the Pitx2-null ventricular cardiomyocytes reveals the lack of an oriented elongation and a loosely arranged myofibrillar apparatus; these cellular features are consistent with an immature, embryonic status, thus suggesting that Pitx2 could exert some role in promoting the establishment of the mature
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cytoarchitecture in ventricular cardiomyocytes. In line with this hypothesis, the ventricular region where the gene is not expressed, although showing clear features of myofibrillar alignment, seem to have a lower degree of cellular maturation if compared to the Pitx2-positive regions. In order to understand if Pitx2 action on cardiomyocytes maturation was a general activity or if it was restricted to the ventricular chambers, we compared by confocal analysis left atrial wt and cTP ko cardiomyocytes, which in wt hearts express Pitx2. Left atrium myocardial cells appeared well differentiated in term of cellular elongation and myofiber organization both in the wt and in the ko samples (Figure 18D); this shows that Pitx2 expression affects cardiomyocytes maturation selectively in the ventricular but not in the atrial cells.

Figure 18: Pitx2 promotes ventricular cardiomyocytes maturation
Confocal immunofluorescence staining for the sarcomeric protein α-actinin (in red) and nuclei (in blue) of wt and cTP ko ventricular sections. The ventricular subregions where the stainings have been performed are indicated in the heart model, where in blue and the white are respectively depicted the regions expressing or not expressing Pitx2. Pictures taken from Pitx2-positive or -negative regions are respectively in a blue or gray frame A, B: In Pitx2-positive ventricular regions the cTP ko cardiomyocytes show a reduced cellular elongation and myofibrillar parallel alignment compared to the wt C: WT cells from ventricular Pitx2-negative regions are less differentiated than ventricular cells expressing the gene and are comparable to cTP ko cells from corresponding regions. D: In left atrial sections, where Pitx2 is expressed, cTP ko cardiomyocytes do not show an impairment in cellular maturation. la,ra: left and right atrium; lv,rv: left and right ventricle.
In conclusion, the characterization of the cTP ko ventricular phenotype revealed a severe interventricular septal defect. This was accompanied by a topological alteration of markers delineating the developing His bundle and bundle branches, but it did not result into a functional impairment of this components as assessed by an optical mapping investigation. On the other hand, at cellular level, a confocal analysis have been performed, revealing the Pitx2-null ventricular cardiomyocytes present a delay in the myofibrillar alignment and lack oriented elongation compared to corresponding wt cells expressing Pitx2. These observations suggest that Pitx2 can contribute to promote cardiomyocytes maturation; this action was selectively exerted in ventricular but not in atrial cardiomyocytes.
5. DISCUSSION

5.1 THE EARLY, MYOCARDIAL ROLE OF PITX2
The crucial role of Pitx2 in the heart has clearly been shown by the complex phenotype of Pitx2 null mice (see section 1.2.6), which includes RAI, VSD, TGA, DORV, common atrioventricular canal (CAVC, in which there is a common opening between all the cardiac chambers), and persistence of truncus arteriosus (PTA), connecting aorta and pulmonary artery during early development (Gage et al., 1999; Liu et al., 2001 and 2002). This phenotype can be the result of Pitx2 early action in the left cardiogenic precursors and/or of a subsequent action in the myocardium.

We show here that almost the complete Pitx2 null cardiac phenotype, including RAI, is recapitulated by conditional deletion of the gene from cardiomyogenesis onset with cTnT Cre mice. Therefore, Pitx2 is required not in the cardiogenic precursors but within the myocardium itself. Our conditional Pitx2 mutant mice present a homeodomain deletion (Gage et al., 1999), resulting in loss of the three gene isoforms; however, given the exclusive myocardial Pitx2c expression (Liu et al., 2001) we conclude that loss of this isoform is responsible for the observed phenotype. In the cTP ko mice we did not detect CAVC and PTA, therefore a residual action of Pitx2 is still required in precardiac cells at the venous and arterial poles.

A previous Pitx2 conditional ko with α-MHC Cre driver mice had outlined a continuous myocardial requirement of the gene for cardiac remodelling, but only ventricular defects were found in that model whereas atrial abnormalities, including RAI, were not present (Tessari et al., 2008). α-MHC Cre is not expressed in atrial or outflow tract precursors at the onset of myocardial differentiation, but only from E8.5-9.0 onwards; Therefore, the left atrial morphological identity is acquired in the time window before α-MHC Cre activation.

Overall, our results show that the absence of Pitx2 from the left sinoatrial region results in a bilateral “right” morphological phenotype, therefore Pitx2 is necessary and sufficient to confer molecular and morphological “leftness” to it, whereas “rightness” seems to be the default state. Moreover, by comparing the cTP ko phenotype with the previous murine models it comes out that left identity acts by patterning cardiomyocytes, not the cardiac precursors, and is acquired very early in development: immediately after the onset of cardiac differentiation.

5.2 PITX2 REPRESSES A NODAL GENETIC PROGRAM IN THE PULMONARY AND CAVAL VEINS MYOCARDIUM: IMPLICATIONS FOR ADULT HEART
The systemic venous tributaries and the pulmonary veins originate from distinct cardiac lineages, respectively from the Nkx2.5(-)/Tbx18(+) sinus venosus myocardium and the Nkx2.5(+)/Isl1(+) secondary
heart field (SHF) (Christoffels et al., 2006; Mommersteeg et al., 2007). From the outset of their myocardial differentiation and throughout all the embryonic development, the myocardial components of the systemic and pulmonary veins are fundamentally different, in particular with respect to their patterning into working or primary myocardium (section 4.2.1.2).

In early development the sinus venosus myocardium, located in the caudal region of the linear heart tube, broadly expresses the essential autorhythmic channel Hcn4 and works as the leading pacemaker of the primitive heart (Mommersteeg et al., 2007); both Hcn4 expression and the pacemaker activity are eventually restricted to the sinoatrial node (SAN), whereas, starting from E14.5, the rest of the sinus venosus-derived myocardium acquires a working phenotype as a consequence of specific Hcn4 transcriptional repression and up-regulation of fast conducting connexins (Mommersteeg et al., 2007). On the contrary, the pulmonary myocardium has a working profile from its initial onset onwards and, under normal condition, never attains a primary (or rather nodal) phenotype (Soufan et al., 2004).

In our cTP ko model we describe that the loss of Pitx2 expression leads to a transcriptional deregulation in both the pulmonary and caval veins myocardium, which respectively maintains and de novo attains the expression of Hcn4, encoding an ion channel potentially sufficient to provide automaticity. The identity shift of both the venous myocardial sleeves to a nodal-like phenotype is consistent with the electrophysiological data assessed by optical mapping. Our analyses have shown that mid fetal hearts present a heterogeneous profile of pacemaker activity originating within all the venous pole, thereby uncovering some degree of functional plasticity at least in these ex-vivo conditions. Additionally, our results indicate that this venous-wide capacity to generate the electrical impulse can be modulated by Pitx2. In wt hearts, expressing Pitx2 on the left, pacemaker activity was mainly restricted to the right side, where the SAN is located. This right-sided dominance was much less pronounced in the cTP ko, where a novel functional SAN could be mapped, whereas the intermediate functional properties in the cTP hets were surprising: they are morphologically normal, therefore their intermediate functional behaviour must be solely due to dose-dependent modulation of venous molecular properties. The observation of a left pacemaker potential independent from a left SAN suggests the importance of a correct Pitx2 gene dosage for repressing the potential pacemaker activity of cardiac venous pole, so that it can be progressively confined exclusively to the SAN.

Overall, the in situ hybridization and the optical mapping experiments highlight the relevance of Pitx2 function not only for the regional morphogenesis of the heart (during the development or the asymmetric patterning of specific anatomical structures) but also in the fine modulation of the transcriptional properties of these cardiac regions (in association with a morphogenetic action and/or independently from it).
The conversion of pulmonary and caval veins to a nodal phenotype and the pacemaker activity detected at the venous pole of cTP ko hearts acquire medical interest in the light of recent findings suggesting a role for Pitx2 in atrial fibrillation (AF), the most common cardiac arrhythmia (Gudbjartsson et al., 2007; Lubitz et al., 2010; see the section 1.2.3). In this pathological condition, rapid and irregular atrial activity overrides SAN function, often resulting in irregular impulse conduction to the ventricles and death. The underlying pathophysiologic mechanisms of atrial fibrillation are not completely understood; in general, atrial fibrillation onset is triggered by the presence of reentry circuits or triggered and/or ectopic pacemaker activity (Allessie et al., 2001; Nattel et al., 2002); in particular, cardiomyocytes of the pulmonary and caval veins myocardium have been shown to be origin of ectopic focal activity (Haïssaguerre et al., 1998; Katritsis et al., 2004). Moreover, once initiated AF itself induces structural and electric remodelling sustaining its maintenance (Dobrev et al., 2010); therefore, even small morphological or molecular alterations making the venous tissue prone to generate ectopic beats can trigger pathophysiologic changes that stabilizes AF. In this line, the ectopic expression of the pacemaker ion channel Hcn4 in the pulmonary and caval vein myocardium of cTP ko mice suggests that Pitx2 disregulation can potentially trigger AF onset by inducing arrhythmogenic foci in these regions.

5.3 PITX2 ROLE IN THE VENTRICLES: AN OPEN ISSUE

Like the sinoatrial region, the ventricular chambers are molecularly patterned along the left-right axis (Campione et al., 2001), with Pitx2 delineating the left lineage on the ventricular ventral side (see section 1.2.4 and figure 4). As discussed above, the study has shown that early Pitx2 expression in myocardial cells of the sinoatrial region is sufficient to confer them “leftness”, since the loss of the gene generates isomeric sinoatrial structures; however, in cases of cardiac isomerism, one might expect not only the atria but also the ventricles to be affected. Nevertheless, left-right differences (which become dorso-ventral after the looping process) are less obvious in ventricles and have never been investigated and/or described: ventricular morphology does not exhibit clear differences detectable as left or right structures and, besides Pitx2 itself, additional molecular markers delineating the left-right patterning are not known at the moment.

Our morphological characterization did not highlight dramatic alterations of shape, atrioventricular connections and chambers growth but allowed to detect septal and ventriculo-arterial alignment defects. The morphology of a tissue is the final readout of fine regulated cellular properties; in particular, cardiac morphogenesis requires a fine and coordinated control of regional cell proliferation, directional cell division, and growth (Meilhac et al., 2004; Soufan et al., 2006). Therefore, we decided to move our investigation at the cellular level in order to understand if Pitx2 could differentially affect some of this cellular features and/or activity which ultimately led to the cTP ko morphological defects. In line with this,
we found that cTP ko ventricular but not atrial cardiomyocytes presented an impairment and/or a delay in the cellular elongation and myofibrillar alignment, suggesting that Pitx2 expression may promote cardiomyocytes maturation specifically in ventricular and not in atrial cells; this action might drive the ventricular remodeling process, which is defective in the cTP ko embryos. We cannot rule out that other cellular functions or properties can be modulated by Pitx2 affecting cardiomyocyte cytoarchitecture, thereby additional investigation will be required to evaluate potential additional Pitx2 actions on proliferation, motility, adhesion or contraction.

At last, the selective cellular action of Pitx2 in ventricular and not in atrial cells is consistent with previous works describing Pitx2 multiple morphogenetic effects in multiple organs and in distinct regions of the same organ (Gage et al., 1999; Piedra et al., 1998) Our results support the idea of a highly regionalized action following a “modular” framework, with distinct and independent pathways of transcriptional activation for each distinct cardiac region of its expression domain. The molecular mechanisms driving the multiplicity of Pitx2 action are at the moment unknown: Pitx2 target specificity in vivo might depend on DNA binding specificity of its related homeodomains, but it is likely modulated by interactions with tissue- and cellular-specific accessory proteins through corresponding interaction domains.
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