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BIOLOGIA MOLECOLARE APPLICATA

Indagine su marcatori delle cellule staminali e delle cellule progenitrici cardiache nel cuore di maiale durante lo sviluppo embrionale.

Investigating cardiac stem cells and progenitor markers in the developing pig heart.

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ITALIAN ABSTRACT

Nei mammiferi lo sviluppo del cuore è un processo complesso che coinvolge differenti popolazioni cellulari e una fitta rete di geni regolatori. Tale processo è ancora largamente sconosciuto nella specie umana, visto che gli embrioni umani sono difficili da ottenere per fare ricerca. La maggior parte delle conoscenze a proposito di questo argomento, proviene da ricerche fatte sui topi, però sono state riscontrate alcune differenze tra il modello umano e quello murino.

Negli ultimi anni il modello suino è statop proposto per sostituire quello murino, le ragioni di questa scelta sono molteplici, prima di tutto l'embrione suino sviluppa come il disco piatto degli embrioni umani e non con la forma cilindrica negli embrioni di topo. In secondo luogo, i maiali sono più simili agli umani in termini di dimensioni e sono già stati usati come modello nelle esrcitazioni pratiche in chirurgia. Una terza e importante considerazione riguarda il fatto che gli embrioni di maiale sono semplici da ottenere.

Considerato che la letteratura scientifica, a proposito dello sviluppo del cuore suino è molto limitata, sono stati definiti gli stadi più importanti della differenziazione cardiaca negli embrioni suini particolare attenzione è stata

posta ai seguenti stadi di sviluppo embrione: (E)15, E16, E18, E20, E22 ed E24. Dopo aver inclusoin blocchi di paraffina, gli embrioni agli stadi selezionati, gli aspetti anatomici dello sviluppo cardiaco suino sono stati caratterizzati utilizzando la tecnica istologica mediante la colorazione ematossilina ed eosina (HE) sulle sezioni ottenute al microtomo.

Inoltre, è stata utilizzata la tecnica dell'immunoistochimica per analizzare i seguenti marker allo scopo di identificare popolazioni cellulari differenti: cellule del mesoderma cardiaco (PDGFR-α, FLK1), cellule progenitrici cardiache (ISL1, NKX2.5) e cardiomiociti maturi (CTNT, CX43, MYHC-B).

I risultati ottenuti hanno permesso di comparare eventi temporali e mofologici chiave con corrispondenti stadi di sviluppo nel cuore dell'uomo e del topo. Quando l'espressione dei marker cardiaci specifici viene paragonata sono state evidenziate più similitudini tra uomo e suino che tra uomo e topo. Per esempio, ISL1 è espresso prima di NKX2.5 ed è presente in stadi di sviluppo avanzati. Inoltre, l'espressione di NKX2.5. Inoltre questa espressione èsovrapponibile a quella di FLK1 e CTNT. Sono stati ottenuti dati di espressione che corrispondono con entrambi i modelli sperimentali e che confermano alcuni lavori precedenti, come FLK1 espresso nell'epicardio. Al contrario, l'espressione di CTNT inizia allo stadio di sviluppo successivo a quello corrispondente nel topo, ma questo dato non può essere confrontato con il

modello umano visto che non ci sono dati a proposito degli embrioni umani in stadi di sviluppo così precoci. Questo studio rafforza i dati di lavori precedenti che riportano NKX2.5 ed ISL1 espressi in entrambi gli "heart fields". Inoltre, suggerisce CTNT e MYHC-β come buoni marker di cardiomiociti, e la conseguente individuazione delle strutture cardiache durante lo sviluppo.

I risultati ottenuti sono in preparazione per essere sottomessi alla rivista scientifica Stem Cells and Development.

ABSTRACT

The development of mammalian heart is a complex process, involving the integration of different cell populations and gene regulatory networks. The process is still largely unknown in humans, since early human embryos are difficult to obtain. Most knowledge has been derived from mice, however, there are most probably differences between these two species.

In the last years, pig model is increasingly used to replace the mouse for manifold reasons, first of all pig embryos develop as flat discs as human embryos, alike mouse egg 'cylinders'. Secondly pigs are more similar to human

with regards to size and they are being used as models already, e.g. doctors practice surgeries on pigs. Third, pig embryos are easy to obtain.

Given that little literature is available on pig heart development, important steps of cardiac differentiation of pig embryos to occur at embryonic day (E)15, E16, E18, E20, E22 and E24, were defined. After embedding the collected specimens in paraffin blocks, cardiac development was characterised by performing hematoxylin and eosin staining (HE staining) on the obtained paraffin sections. Furthermore, immunohistochemistry was performed on paraffin sections to analyse the following markers to identify different cell populations: cardiac mesoderm cells (PDGFR-α, FLK1), cardiac progenitor cells (ISL1, NKX2.5) and mature cardiomyocytes (CTNT, CX43, MYHC-B).

The obtained results allowed to compare temporal key morphological events by corresponding human and mouse developing heart stages. When comparing specific cardiac markers expression more similarities were noticed between pigs and humans, than between mice and humans. For example, ISL1 was expressed before NKX2.5 and was present at later stages of development. Furthermore, NKX2.5 expression overlapped with FLK1 and CTNT. On the other hand, the obtained results matched with both models and confirmed some preceding works, like FLK1 expressed in the epicardium. However, CTNT appearance after the corresponding stage in mouse can’t be compared with

human model, since there is no literature about early human embryo stages. Notably, this study strengthens previous data reporting NKX2.5 and ISL1 to be expressed in both heart fields. Moreover, it outlines CTNT and MYHC-β as good cardiomyocyte markers delineating the cardiac structures during the development.

The obtained results will be submitted to the Stem Cells and Development journal.

INTRODUCTION

1.1.1 Mammals cardiac development

In the early stages of development within the uterine cavity, uterine glands secret fluid to feed the embryo. Along with the embryo's growth, a circulatory system takes shape to supply the embryo's need of nutrient distribution and metabolite removal (Hyttel, 2010). The circulatory system includes the heart, which is the first functional organ. Heart formation starts from the embryonic disk, where lateral cardiogenic mesoderm froms ventrally along the embryonic midline to create the cardiac tube (Jensen et al. 2013). This further undergoes dextral looping to acquire the appropriate left–right asymmetry. The looping

heart tube can be further subdivided into four chambers and the endocardial cushions develop into the valves. Together with development of the great vessels, this enables unidirectional bloodflow through the chambers (Clowes et al. 2014). This process is described in more detail in the following sections.

1.1.2 Cardiac tube formation

In the anterior lateral splanchnich mesoderm, clusters of hemangioblasts canalise to create a fused tube. This horseshoe-shaped structure, also called the cardiogenic field, is located in the antero-lateral portion of the embryonic neural plate (Hyttel, 2010). The horseshoe-shaped cavity is lined by endothelial cells forming the endocardium. Mesenchyme-derived myoblasts surround the endocardial tube composing the myocardium. The intra-embryonic coelom overlying the cardiogenic field develops into the pericardial cavity and its visceral mesoderm forms the epicardium to complete the cardiac tube (**Figure 1;** Hyttel, 2010).

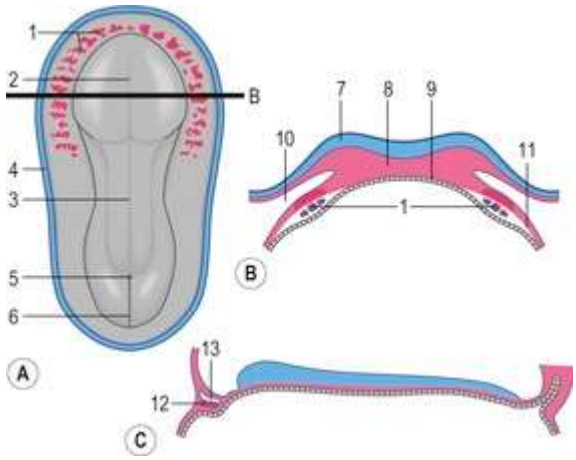

Figure 1. Cardiogenic field formation. A: Dorsal view of an embryo after removal of the amniotic folds. The cardiogenic field (1) is seen as a horseshoe-shaped structure anteriorly. 2: Neural plate; 3: Neural groove; 4: Cut edge of chorioamniotic fold; 5: Primitive node; 6: Primitive streak. B: Transverse section of embryo at the line 'B'. 7: Neural ectoderm; 8: Mesoderm; 9: Endoderm; 10: Intra-embryonic coelom; 11: Visceral mesoderm; C: Median section through the embryo. 12: Cardiogenic field; 13: Pericardial cavity. (Hyttel, 2010).

Posterior to the cardiogenic field, a bilateral assembly of angioblast clusters leads to the formation of two dorsal aortae. These bilateral tubes are lined by endothelial cells. The horseshoe-shaped cardiac tube which is enclosed in its pericardial cavity undergoes a 180 degree translocation from an anterior to a ventral position, with the resulting inversion of the heart tube with an anterior and posterior extension. Besides the antero-posterior folding, a lateral folding occurs, the embryonic disk lateral regions are moved ventrally, until they encounter and fuse with each other in the midline of the embryo to form a single tube. After this folding, the cardiac tube is positioned ventrally to the anterior portion of the dorsal aortae and the anterior heart tube extensions develop into the two ventral aorta, which become the future outlet of the heart. This strategic

anatomical position allows aortic arches to connect the dorsal and ventral aortae on each side. The future heart inlet can be defined by bilateral collecting systems that drain the arterial system and which connects to the posterior cardiac tube crescent (**Figure 2, B-G;** Hyttel, 2010). At this stage of development, the heart tube enlarges in size and starts to beat pumping blood out into the developing arterial system; receiving venous drainage in return. To evolve into the four-chambered mammalian heart where the body and lung blood circulation systems are separated, the cardiac tube undergoes various processes, including loop formation and a following internal division.

1.1.3 Cardiac loop

Situated in the pericardial cavity, the cardiac tube is connected with the dorsal and ventral mesocardium which deteriorates in a short time period. During its growth, some parts of the cardiac tube expand faster than others and the consequence is a segmented tube with dilations separated by indentations which are connected by narrower channels (Hyttel, 2010). These expanded portions in posterior-anterior order are sinus venosus (SV), atrium (AT), the

ventricle (VE), bulbus cordis (BC) and the truncus arteriosus (TA) (**Figure 2, H, I;** Hyttel, 2010).

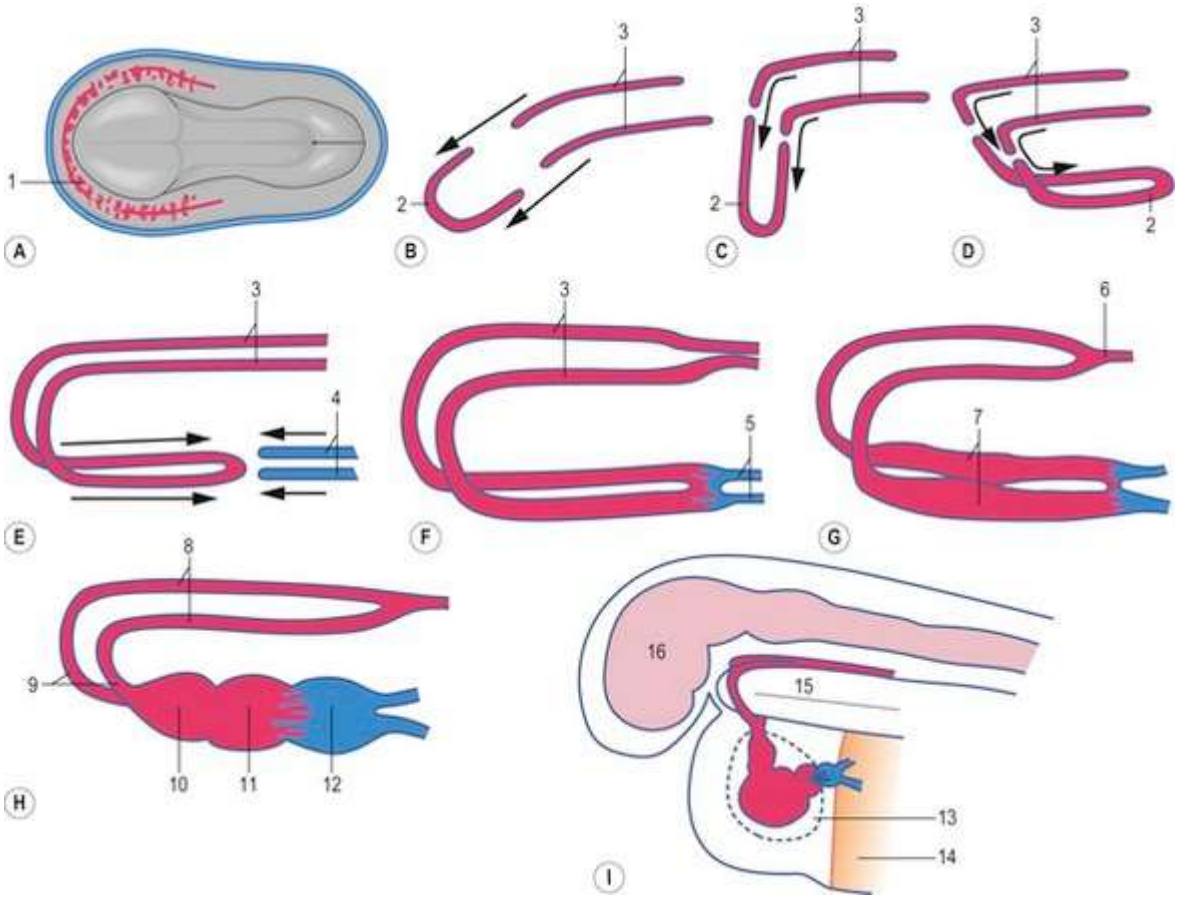

Figure 2. Positioning of the heart and development of the dorsal and ventral aortae. A: Dorsal view of embryo with the cardiogenic field (1) anteriorly. B: With the cranio-caudal folding of the embryo, the cardiac tube (2) is brought caudo-ventrally. The developing dorsal aortae (3) approach the cardiogenic tube. C–E: The cardiogenic tube (2) is brought to a position ventral to the dorsal aortae (3) and the vitelline veins (4) approach the cardiogenic tube. F: The caudal portion of the cardiogenic tube fuses (5) with the cranial portion of the vitelline veins. G: The caudal portions of the dorsal aortae fuse (6) and the two sides of the cardiac tube fuse as well (7). H: The dorsal (8) and ventral (9) aortae have formed, and the developing heart has formed the bulbus cordis (10), ventricle (11) and atrium (12). I: Overview of the position of the developing heart and the dorsal and ventral aortae. 13: Pericardial cavity; 14: Septum transversum; 15: Primitive gut; 16: Brain vesicles. (Hyttel, 2010).

The origin of the TA neural crest cells is particularly noteworthy.

Expanding itself, the cardiac tube outgrows the pericardial cavity and since it is fixed in the pericardium at both ends, the heart tube undergoes a loop with the junction between the VE and BC, resulting in it pointing ventrally.

During this shape changing period the pacemaker cells in the SV set the beating rythm.

Furthermore, this loop event leads to the SV and AT inclusion within the pericardial cavity, positioning the AT dorsal to the VE and the loop takes on an S shape (**Figure 3;** Hyttel, 2010).

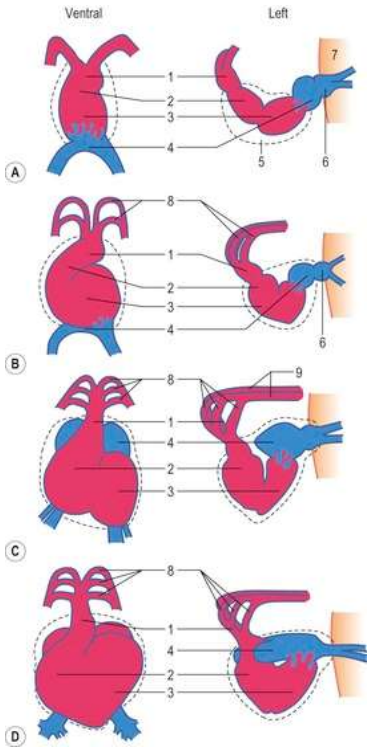

Figure 3. Ventral and left aspects of the segmentation and loop formation of the heart at progressive stages of development (A-D). 1: Truncus arteriosus; 2: Bulbus cordis; 3: Ventricle; 4: Atrium; 5: Pericardial cavity; 6: Sinus venosus; 7: Septum transversus; 8: Aortic arches; 9: Dorsal aortae. (Hyttel, 2010).

1.1.4 Four chambers formation

Three pairs of veins including, the vitelline vein, umbilical and the cardinal veins are connected to the SV, to form the right and left sinus horns. As the right sinus horn merges into the AT, the lateral part becomes the crista terminalis (a crest-shaped borderline between the incorporated sinus and the AT), and the medial part becomes the septum secundum. The cranial vena cava develops from the right sinus horn anterior portion, whereas the right sinus horn posterior portion develops into the caudal vena cava. The SV right part (right sinus horn), incorporated into the AT, go to form the AT smooth-walled region and the original right AT remains as the right auricle, whereas the left part of SV (left sinus horn) develops into the coronary sinus. The pulmonary vein merges into the left part of AT and expands from a single opening to four openings, resulting in the smooth-walled left atrium region. The original left AT forms the left auricle (**Figure 4, 5;** Hyttel, 2010).

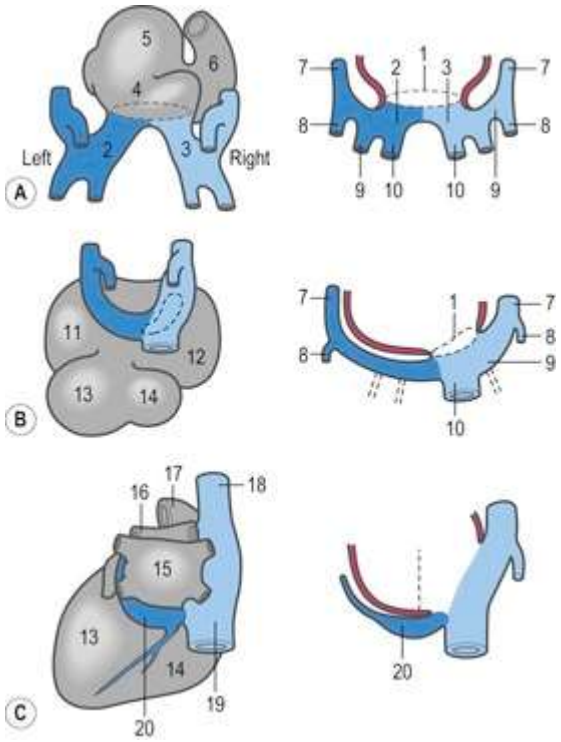

Figure 4. Dorsal aspect of the development of the venous system in the heart region. 1: Sinoatrial opening; 2: Left sinus horn; 3: Right sinus horn; 4: Atrium; 5: Ventricle; 6: Bulbus cordis; 7: Left and right anterior cardinal veins; 8: Left and right posterior cardinal veins; 9: Left and right umbilical veins; 10: Left and right vitelline veins; 11: Left atrium; 12: Right atrium; 13: Left ventricle; 14: Right ventricle; 15: Left atrium; 16: Pulmonary arteries; 17: Aorta; 18: Cranial vena cava; 19: Caudal vena cava; 20: Coronary sinus. (Hyttel, 2010).

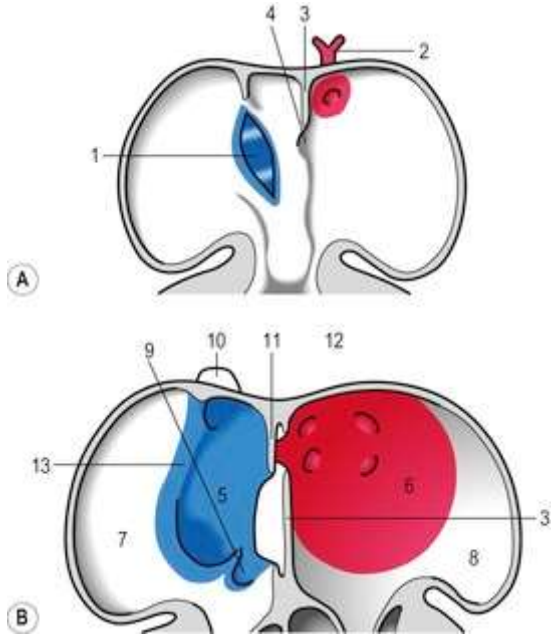

Figure 5. Incorporation of the right sinus horn and the pulmonary veins into the atrium at two stages of development (A, B). 1: Opening of right sinus horn into the atrium; 2: Opening of the pulmonary veins into the atrium; 3: Septum primum; 4: Ostium primum; 5: Incorporated portion of right sinus horn; 6: Incorporated portion of pulmonary veins; 7: Right auricle; 8: Left auricle; 9: Opening to caudal vena cava; 10: Opening to cranial vena cava; 11: Septum secundum; 12: Foramen ovale; 13: Crista terminalis. (Hyttel, 2010).

1.1.5 Four chamber septation

The four chamber division continues to develop. Anterior and posterior thickening occurs along the atrioventricular channel, forming the endocardial cushions. As these grow, they fuse, forming the septum intermedium which divides the channel into right and atrioventricular channels (AVC) (**Figure 6;** Hyttel, 2010).

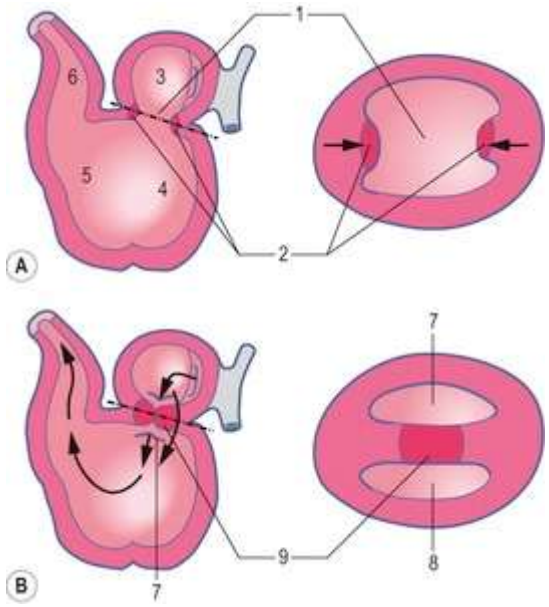

Figure 6. Division of the common atrioventricular canal into left and right canals at different stages of development (A-B). Diagrams to the right are dorsal views of areas indicated by broken lines. 1: Common atrioventricular canal; 2: Endocardial cushions; 3: Atrium; 4: Ventricle; 5: Bulbus cordis; 6: Truncus arteriosus; 7: Right atrioventricular canal; 8: Left atrioventricular canal; 9: Septum intermedium. In 'A' the arrows indicate growth of the cushions, and in 'B' the arrows indicate the direction of blood flow. (Hyttel, 2010).

To complete the AT septation, the septum primum develops from the dorsal AT wall, dividing it into right and left chambers, leaving a small opening, the ostium primum which is a temporary connection between the two chambers that gradually closes. The ostium primum closure is compensated by the ostium secundum formation, which continues the blood flow between the developing right and left AT. Ostium secundum is later closed by the septum secundum, which develops on the septum primum left side, however an opening called the foramen ovale remains throughout the rest of fetal development. Subsequently, the blood flow from the right to the left AT is regulated by a valve established

in the ventral part of the septum secundum, while this latter dorsal portion fuses with the septum primum. The ventricular division occurs with the interventricular septum formation and is created by thickening and fusion of the trabeculated VE and BC walls brought in apposition. As the interventricular septum growth meets the septum intermedium, it divides the VE (future left ventricle) and the BC (future right ventricle) but leaves a temporary opening, the interventricular foramen (**Figure 7;** Hyttel, 2010).

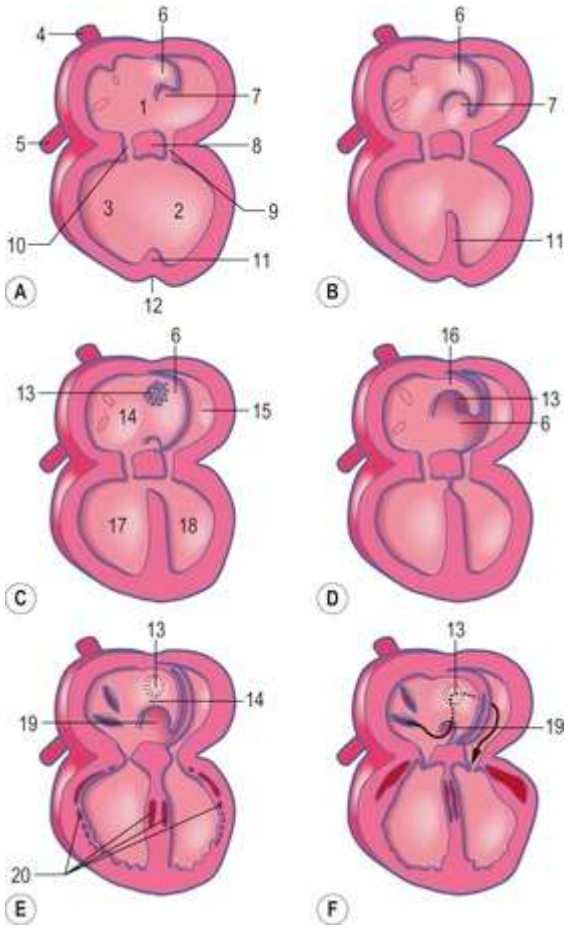

Figure 7. Partitioning of the heart into four chambers. 1: Atrium; 2: Ventricle; 3: Bulbus cordis; 4: Cranial vena cava; 5: Caudal vena cava; 6: Septum primum; 7: Ostium primum; 8: Septum intermedium; 9: Left atrioventricular canal; 10: Right atrioventricular canal; 11: Interventricular septum; 12: Interventricular sulcus; 13: Foramen secundum; 14: Right

atrium; 15: Left atrium; 16: Septum secundum; 17: Right ventricle; 18: Left ventricle; 19: Foramen ovale; 20: Cavitations in myocardium. (Hyttel, 2010).

At this point two valve systems develop to ensure the blood flow direction, including, the atrioventricular valves and the semilunar valves.

Cavitation of the primitive left and right ventricle myocardium allows the formation of muscular cords which attach the valves suspended from the edges of the left and right AVCs. Bicuspid or mitral valves (two valves) are located on the left side, whereas tricuspid valves (three valves) are found on the right. The muscular cords connected to valves are then replaced by the connective tissue, termed the chordae tendineae, whereas papillary mussels develop from the rest of the muscular cords.

The semilunar valves develop as swellings at the outlet of the TA into the ventral aortae during the division of the TA by the growing cushions. Upon completion of the aorticopulmonary septum, the swelling gives rise to three primitive valves in each of the aortic and pulmonary outlets (Hyttel, 2010).

The contraction propagation is allowed by myocardial cells connected through gap junctions. Clusters of the specialized cells form the atrioventricular node and atrioventricular fasciculus which distribute the impulses in the ventricular

walls thanks to Purkinje fibres. Whereas the sinoatrial node is formed subepicardially at vena cava caudalis future opening (Hyttel, 2010).

1.2 Cardiac precursor cells and origin of the heart fields

Accumulated knowledge over the years from scientific discoveries, highlights that heart development depends on the contribution of three spatially and temporally distinct cell populations: cardiac progenitor cells (CPCs), proepicardium and cardiac neural crest cells. The first are originated by cells migrating away from primitive streak, during gastrulation, and going to compose the cardiac mesoderm cells, whithin the splanchnic mesoderm, that will give rise to first and second heart fields (FHF, SHF), the cardiogenic mesoderm regions intended to develop the heart endocardium and myocardium. The second, constituted by proepicardium will form the future epicardium, whereas cardiac neural crest cells have an important role in the heart arterial pole maturation (Brade et al., 2013). Outlining these mechanisms help in understanding heart development better.

1.2.1 *Cardiac mesoderm formation*

Cardiac mesoderm, important to originate FHF and SHF CPCs, derives from numerous signaling pathways, here described in details.

During gastrulation, located in the epiblast, pluripotent stem cells expressing T-Box, transcription factor T (Brachyury) migrate from the posterior to the anterior portion of the primitive streak. These migrating cells are characterized by the helix-loop-helix transcription factor mesoderm posterior 1 (Mesp1), which is the first marker of cardiogenesis specification. Mesp1 repress pluripotency genes, promoting the early phases of cardiogenesis (Paige et al., 2015), allowing the migration of the differentiating cells anteriorly and laterally to the primitive streak to form the cardiac mesoderm (Robert G. Kelly, 2016) (**Figure 9, a;** Clowes et al., 2014). Mesp1 is co-regulated by T-Brachyury and T-box transcription factor eomesodermin (Eomes) (Jelena Tosic et al., 2015).

Cardiac mesoderm specification, whithin the splancnic mesoderm, is promoted by noncanonical WNT ligands such as Wnt11, as well as fibroblast growth factors (FGFs), bone morphogenic protein (BMP) and sonic hedgehog (Shh) from the endoderm. Conversely, it is inhibited by canonical Wnt ligands, including Wnt1, Wnt3a and Wnt8 secreted from the overlying neuroectoderm. Furthermore noggin and chordin from notochord partecipate to this cardiac

specification repression (**Figure 8;** Paige et al., 2015). This cardiac mesoderm induction process allows to originate FHF and SHF.

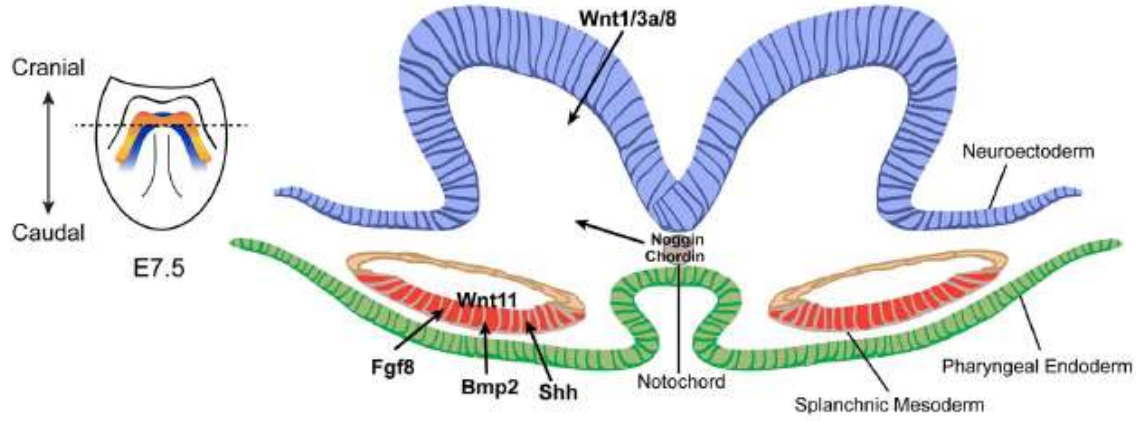

Figure 8. **Regulation of cardiac mesoderm specification.** Shown is a cross-section of an E7.5 mouse embryo detailing the signaling pathways that regulate cardiac specification within splanchnic mesoderm. Factors secreted by the adjacent endoderm that support cardiac mesoderm specification include fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and sonic hedgehog (Shh). In addition, noncanonical Wnt ligands, such as Wnt11, expressed in splanchnic mesoderm also promote cardiac differentiation. Conversely, canonical Wnt ligands, including Wnt1, Wnt3a, and Wnt8, secreted from the overlying neuroectoderm, as well as BMP antagonists noggin and chordin secreted from the notochord inhibit cardiac mesoderm specification, thereby limiting the size of the cardiogenic fields. (Paige et al. 2015)

1.2.2 Cardiac progenitor cells as components of the first and second heart fields

The first cells to migrate, expressing Mesp1, compose the FHF (Sharon L. Paige et al., 2015). These CPCs then migrate towards the midline to create the cardiac crescent. Subsequentely, the same cells differentiate and fuse to form the linear tube (Clowes et al., 2014). Anteriorly and medially to the FHF cells, the SHF develops from a later wave of Mesp1 positive cells from the pharingeal mesoderm, which contrary to the FHF do not differentiate but maintain their cardiac progenitor identity (Paige et al., 2015) (**Figure 9, b, c;** Clowes et al., 2014). The embryonic heart tube developing from both the FHF and SHF consists of two cell layers, the endocardium and myocardium. Several experiments have stated that FHF forms the left VE, most of the atria and part of the right VE, while the SHF gives rise to the right VE, outflow tract (OFT) and part of the atria (Calderon et al., 2016) (**Figure 9, d-f;** Paige et al., 2014). FHF and SHF CPCs can differentiate into multiple cell lineages, cardiomyocytes, smooth muscle cells, and endothelial cells (Calderon et al., 2016; Brade et al., 2013).

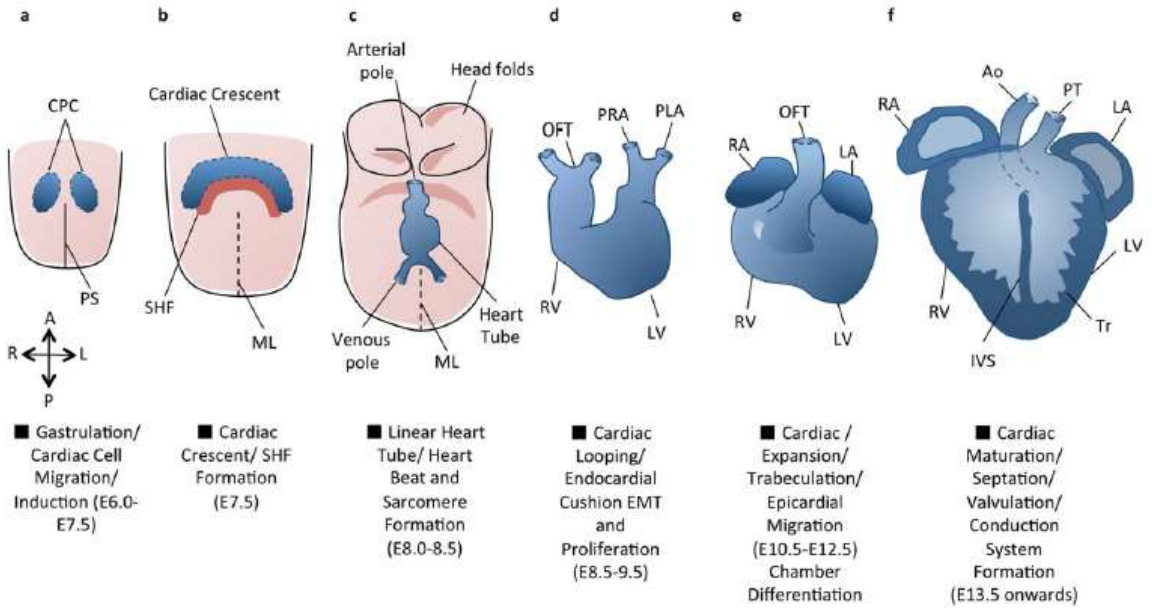

Figure 9. An overview of cardiac development. Cardiac development progresses from the specification of cardiac progenitor cells (a) to the migration of these cells towards the midline to form the cardiac crescent (b). The developing heart then forms a linear tube (c), which undergoes dextral looping to acquire the appropriate left–right asymmetry (d). The heart tube is further subdivided into the four chambers (e), and the maturation of the endocardial cushions into the valves and development of the great vessels provides for unidirectional blood flow through the chambers (f). Adapted from (Buckingham et al., 2005). A=Anterior, Ao=Aorta, CPC=Cardiac Precursor Cells, IVS=Interventricular septum, L=Left, LA=Left Atrium, LV=Left Ventricle, ML=Midline, OFT=Outflow Tract, P=Posterior, PHF= Primary Heart Field, PLA=Primitive Left Atrium, PRA=Primitive Right Atrium, PS=Primitive Streak, PT=Pulmonary Trunk, R=Right, RA=Right Atrium, RV=Right Ventricle, SHF=Secondary Heart Field, Tr=Trabeculae (Clowes et al., 2014).

1.2.3 Proepicardium and epicardium

The outermost layer of the heart called the epicardium is derived from a cluster of cells known as the proepicardium. The proepicardium is a transitory structure that emerges from the coelomic mesenchyme of the septum transversum, close to the venous pole of the linear heart tube at early stages. Proepicardium-derived cells form the epicardium tissue and differentiate into

interstitial fibroblasts embedded in the myocardium, vascular smooth muscle cells and endothelial cells of the coronary vessels, and some myocytes, mainly in the atrioventricular septum. Notably, the epicardium interacts with the underlying myocardium to regulate chamber maturation and ventricular muscle growth (**Figure 10, A, B;** Brade et al., 2013).

1.2.4 Cardiac neural crest

Another important cell population, namely cardiac neural crest cells, migrate from the neural tube dorso-lateraly and reach the caudal pharyngeal arches to continue migrating to the OFT. There, they contribute to heart development, by establishing the aorticopulmonary septum and give rise to the tunica media smooth muscle cells of the arterial pole vessels distal region. Further contributions of the cardiac neural crest cells are the conotruncal region, the interventricular septum and sensory heart innervation (Paige et al., 2015) (**Figure 10, A, C;** Brade et al., 2013).

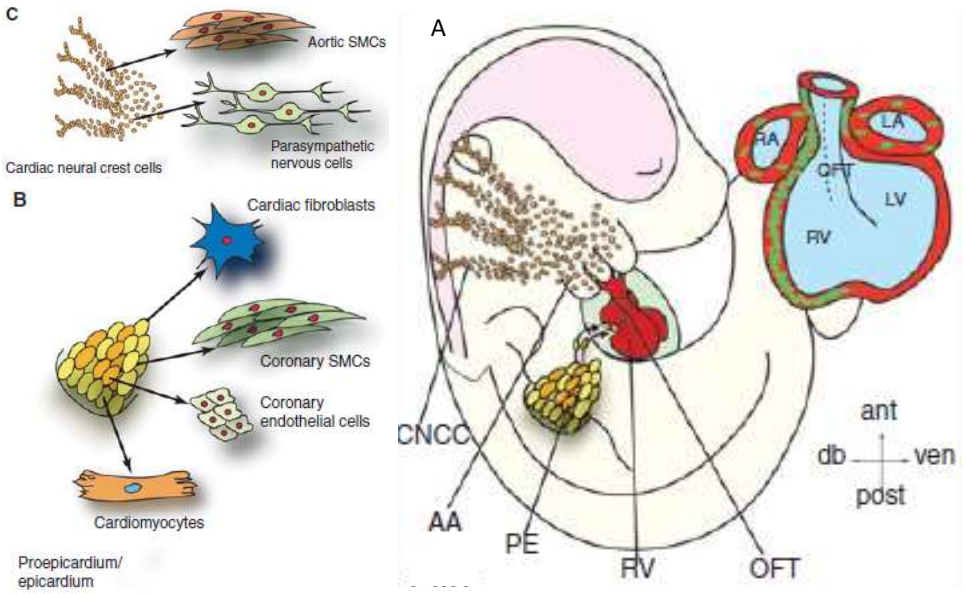

Figure 10. Proepicardium and cardiac neural crest progenitor contributions to different cardiac compartments and cell types during heart morphogenesis in mammals. (A) Cardiac neural crest and proepicardial cells contribute to the heart, which already shows a defined four-chamber morphology, (B) proepicardium/epicardium, and (C) cardiac neural crest cell lineage diversification. AA, Aortic arch; ant, anterior; CNCC, cardiac neural crest cells; do, dorsal; L, left; LA, left atrium; LV, left ventricle; OFT, outflow tract; PE, proepicardium; post, posterior; PRA, primitive right atrium; R, right; RA, right atrium; RV, right ventricle; SMCs, smooth muscle cells; ven, ventral. (Brade et al., 2013).

1.3 An outline of selected cardiac markers

In the last decade, several molecular markers have been discovered to identify heart developmental and the involved cell populations. For this study, a set of markers has been selected for investigation in the developing pig heart (**Table 1**). The following chapter describes these markers and the knowledge that has been generated with several model organisms and a manuscript prepared for submission to an international peer-reviewed journal.

Marker	Function	Location
PDFGR-α	A cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family. These growth factors are mitogens for cells of mesenchymal origin.	Plasma membrane, nucleus
FLK1	Kinase insert domain receptor, is a type III receptor tyrosine kinase. It functions as the main mediator of VEGF-induced endothelial proliferation, survival, migration, tubular morphogenesis and sprouting.	Membrane
NKX2.5	Homeobox-containing transcription factor. This transcription factor functions in heart formation and development. Mutations in this gene cause atrial septal defects with atrioventricular conduction defects.	Mainly in the nucleus, less in the cytoplasm
ISL1	Member of the LIM/homeodomain family of transcription factors. The encoded protein binds to the enhancer region of the insulin gene, among others, and may play an important role in regulating insulin gene expression.	Nucleus
CTNT	Tropomyosin-binding subunit of the troponin complex, which is located on the thin filament of striated muscles and regulates muscle contraction in response to alterations in intracellular calcium ion concentration.	Cytoplasm
CX43	Is the major protein of gap junctions in the heart that are thought to have a crucial role in the synchronized contraction of the heart and in embryonic development.	Cell membrane, endoplasmic reticulum
MYHC-β	Beta (or slow) heavy chain subunit of cardiac myosin. It is expressed predominantly in normal human ventricle.	Cytoplasm

Table 1. Ivestigated markers in the developing pig heart.

1.3.1 Cardiac mesoderm progenitor markers

Cardiac mesoderm progenitor cells are multipotent and can differentiate into tri-lineage cell populations, including, cardiomyocytes, endothelial and vascular smooth muscle cells. Co-expression of two cell-surface proteins,

PDGFR-α (Platelet-Derived Growth Factor Receptor, Alpha Polypeptide) and FLK1(Fetal liver kinase, Vegfr2, Kdr), indicates a mesoderm population specified to the cardiac lineage (Calderon et al., 2016). FLK1 is a tyrosine kinase receptor for VEGF (vascular endothelial growth factor) and although it is essential to establish vascular and hematopoietic systems in the embryo, it has been found to be expressed in endothelial cells, but not in most hematopoietic cells at later stages of development (Ishitobi et al., 2011). PDGFR-α is a cell-surface receptor tyrosine kinase for platelet-derived growth factors and is reported to be broadly expressed in primitive endoderm and mesoderm derivatives thorough embryogenesis. PDGFR-α mRNA was detected in many areas of mesenchyme derivatives, including the somites, limb bud and branchial arches (Quian et al., 2017).

1.3.2 FHF and SHF cardiac progenitor markers

Genetic lineage tracing studies with mice report NKX2.5 (NK2 Homeobox 5) as a marker that identifies both FHF and SHF, while ISL1 (ISL LIM Homeobox 1) is restricted to the SHF. This suggests that cardiomyocytes and smooth muscles cells are originated in the FHF by NKX2.5 positive cells and in the SHF by NKX2.5-ISL1-positive cells, while in the same SHF, endothelial cells derive from FLK1-ISL1-positive cells (Brade et al., 2013) (**Figure 11;** Paige et

al., 2014). This genetic tracing has been confirmed by single-cell RNA sequencing analysis (Xiong et al., 2020).

However, some research indicate that molecular differences observed between FHF and SHF are a result of Cre-based fate-mapping studies, in which partial recombination of Cre reporter genes can complicate interpretation of Cre fate-mapping experiments (Ma et al., 2008). Consistent with this idea, they report both markers characterize either heartfields (Ma et al., 2008).

ISL1 is described to be highly expressed in proliferative cells, while NKX2.5 binds an Isl1 enhancer and represses ISL1 promoting the progenitor cells diffentiation (Calderon et al., 2016).

ISL1 belongs to a family of transcription factors, known to be located in the nucleus, that binds and regulates promoter region of the insulin gene, among others, and may play an important role in regulating insulin gene expression, furthermore it has been found expressed in multiple tissues and cell types, including the central and peripheral nervous system, neural retina, inner ear, pharyngeal mesoderm, endoderm and their derivatives (craniofacial structures, thymus, thyroid gland and trachea), cardiovascular system (cardiac outflow tract, carotid arteries, umbilical vessels, sinoatrial node and atrial septum), gastrointestinal system (oral epithelium, stomach, pancreas, mesentery) and hindlimb (Zhuang et al., 2013). In literature ISL1 expression is limited at early

stages of development to be then lost rapidly, with exceptional positive cells found in adult heart (Iancu et al. 2015).

NKX2.5 is an homeodomain transcription factor located in the cellular nucleus, expressed in the heart progenitors thanks to a tussue specific enhancer (Iancu et al. 2015). This marker has been further found expressed in the developing foregut, thyroid, pharynx, stomach, and spleen (Moses et al, 2001). In contrast to ISL1, NKX2.5 is not downregulated at the end of development (Iancu et al. 2015).

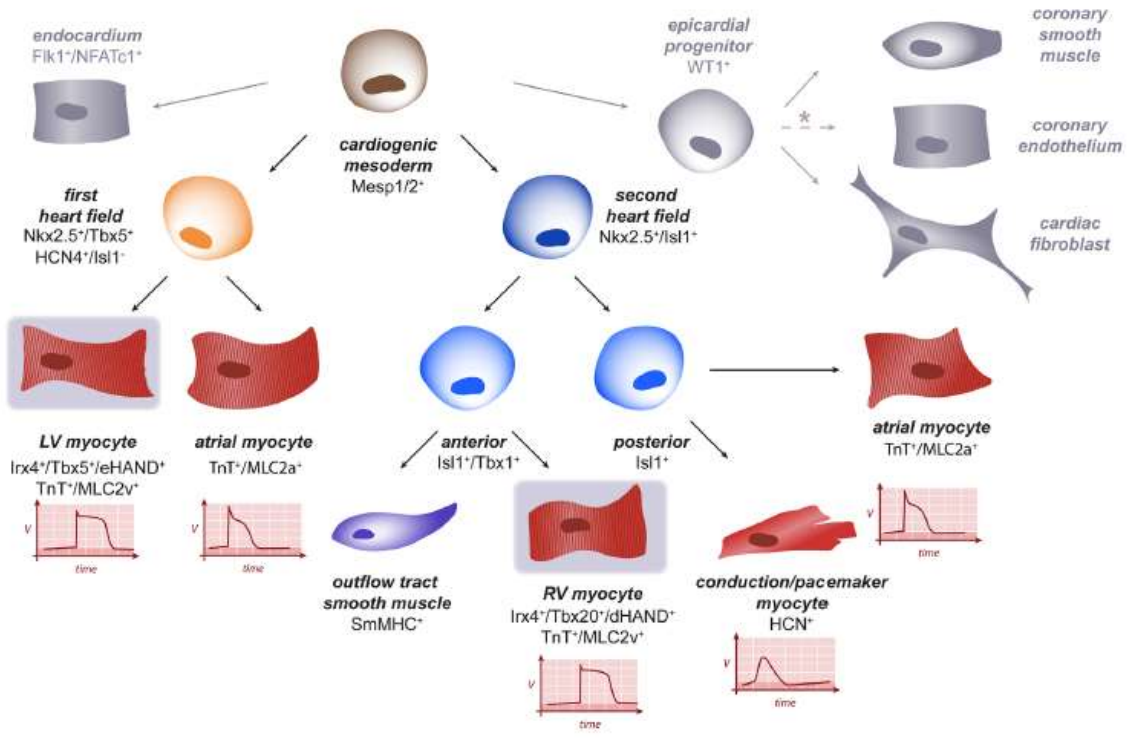

Figure 11. **Schematic of cardiovascular lineage diversification.** The specification of cardiomyocytes in the first heart field (FHF) and second heart field (SHF) is shown in the context of other cardiac cells that are also derived from a common cardiogenic mesoderm

progenitor. In particular, Isl1 expression distinguishes the FHF and SHF. Comparison of action potentials for mature myocytes reveals the range of function generated from each heart field. *Developmental origin of the coronary endothelium is an active topic of investigation. Whereas some evidence points to partial contributions to the coronary endothelium from the epicardium, other sources, such as the endocardium and the sinus venosus, have also been reported. LV indicates left ventricle; and RV, right ventricle. (Paige et al., 2015)

1.3.3 Cardiomyocyte markers

Cardiomyocytes can be identified thanks to the high expression of important structural genes, like myofibrillar proteins MYHC-β (Myo7), CTNT (TNNT2, Troponin T) and the surface marker CX43 (Connexin 43) (Forough et al., 2011). CTNT is the tropomyosin-binding subunit of the troponin complex and plays a core function in the regulation of striated muscle contraction in response to alterations in intracellular calcium ions concentration. Its subcellular location is cytoplasmic and data from mice reveals CTNT expression at early stages of development when the linear tube is formed and not as restricted as that in the adults (Wang et al. 2001).

MYHC-β is the β heavy chain subunit of cardiac myosin, located in the cytoplasm. In mice this marker begins to be expressed after linear tube formation and as soon as the ventricular chambers take form, it becomes restricted in ventricular cardiomyocytes. Contrary to MYHC-β persistence in

adult human VE, in mice it is almost completely replaced by MYHC-α isoform after birth (Morkin, 2000).

CX43 is the major protein of gap junction, situated on the cardiomyocytes membrane in the intercalated disks of cardiac muscles, responsible in the synchronized developing heart contraction. In mice it is present throughout early cardiac development and increases in abundance during late gestation in both atrial and especially in working ventricular cardiomyocytes (Giovannone 2012). In addition CX43 has been found expressed in brain, neural tube, prevertebra, limb and is associated with developmental processes mediated by inductive interactions, such as that of the eye, otic vesicle, kidney, and the branchial arches (Ruangvoravat et al., 1992).

1.4 Why use pig as model and aim of the study

Lately, the use of the pig model in research to study human embriology is increasing. The reason is that mouse embryos develop as 'cylinders', while pig embryos develop as flat discs similar to human embryos (Kobayashi et al., 2017) and *"there are dramatic species-specific differences between human and mouse heart development"* (Cui et al., 2019).

Therefore, the aim of this study was to investigate markers of specific cell populations (PDGFR-α, FLK1, ISL1, NKX2.5, CTNT, CX43, MYHC-B) in the developing pig heart, at different developmental stages, E15, E16, E18, E20, E22, E24, E70 and compare their expression with literature data from mice and human.

2 Materials and methods

2.1 Pigs embryos and fetal pig heart

Pig embryos were obtained at day E15, E16, E18, E20, E22, E24 of gestation (three embryos per day) following insemination of Landrace/Yorkshire sows (twice within 24hrs) using semen from a Duroc boar from a local pig production farm (Roskilde, Denmark). The sows were slaughtered at the above predefined days calculated after the second insemination and the embryos were collected within 30 min after slaughter and transported at 37°C to the University of Copenhagen, where the fetuses (clinically dead from asphyxiation during transport) were isolated by umbilical excision from their fetal sacs. The E70 pig heart was dissected from a single fetus. The embryos and heart were fixed

in 4% paraformaldehyde (PFA) in PBS at 4ºC overnight and stored until use in PBS at 4ºC containing 0.02% sodium azide.

2.2 Tissue preparation and Haematoxylin & Eosin staining

The embryos and heart were paraffin embedded in a tissue processor (Thermo Fisher Scientific Citadel 2000), where they were dehydrated using an increasing ethanol concentration and then "cleared" in Estisol220 before infiltration with paraffin. Subsequently, the specimens were embedded in paraffin wax (Paraffin wax blended with synthetic polymers, VWR). From the paraffin blocks saggital sections (5 μm) were cut using a microtome (SM2000R, Leica). The sections were mounted onto glass slides and stored at 4ºC. For initial histological examination of the tissue, sections were deparaffinized, rehydrated and every 5th or 10th section (depending on embryo size) was stained with haematoxylin-eosin.

2.3 Immunohistochemistry

Paraffin sections were heated at 60°C for 40 min and rehydrated through xylene and ethanol washing. Slides were dipped twice for 10 min each in xylene (VWR) followed by decreasing ethanol concentrations : twice 5 min in 99% ethanol, 3 min in 96% ethanol and 3 min in 70% ethanol.

Immunohistochemistry was performed using two different protocols depending on the species of the antibody used. Primary antibodies: FLK-1 Rabbit polyclonal IgG (1:50, Abcam, Ab45010), NKX2-5 Rabbit polyclonal IgG (1:25, Thermofisher, PA5-49431), ISl-1 Rabbit polyclonal IgG (1:300, Merck Millipore, AB4326), CX43 Rabbit polyclonal IgG (1:1000, Abcam, Ab11370), CTNT Mouse monoclonal IgG2b (1:400, Abcam, Ab10214), MYHC-B Mouse monoclonal IgG1 (1:4000, Abcam, Ab11083), Negative Mouse IgG1 (1:40, DAKO, x0931), Normal Rabbit IgG (1:2000, DAKO, x0936), Negative Mouse IgG2b (1:40, DAKO, x0944), Secondary Antibody Biotinylated goat anti-rabbit (1:250, DAKO, E0432), Third Layer Streptavidin/HRP (1:500, DAKO, P0397, lot. 00032671, 0.83g/L) (Fig. 6). For monoclonal mouse antibodies we used the UltraVision™ Quanto Detection System HRP DAB (Thermo Fisher Scientific). Briefly, rehydrated sections were washed for 5 min in Milli-Q water (MQ) and antigen retrieval was performed by immersion three x 5 min in boiling 0.01 M sodium citrate solution (PH 6, Millipore Sigma). Sections were then washed in 4oC PBS and placed on ice. The Ultravision Hydrogen Peroxide Block was then applied for 10 min, followed by washing in PBS for 5 min and incubation in the Block for another 5 min. Protein block was decanted prior to a 30 min incubation with primary antibodies. The sections were then washed in PBS for 5 min and incubated with Primary Antibody Amplifier Quanto for 10

min followed by another PBS wash for 5 min. In the last step the sections were washed 3 min in PBS, then 3 min in MQ and 3 min in PBS. The sections were then stained with DAB+ Substrate Chromogen system (DAKO) for 5 min. For the polyclonal rabbit antibody, sections were kept 5 min in PBS following rehydration and permeabilized with 0.1% TritonX-100 (MilliporeSigma) for 30 min. The sections were washed twice for 5 min in PBS prior to antigen retrieval (performed as above). The sections were subsequently incubated with 3% H202 in water for 10 min. After two washes in PBS for 5 min, the slides were blocked with 10% Normal Goat Serum (NGS, DAKO) in 1% Bovine Serum Albumin (blocking buffer) (BSA, Millipore Sigma) in PBS for 1 h, washed shortly in PBS and then incubated with primary antibody diluted in blocking buffer overnight at 4°C. The next day, after two washes for 5 min in PBS, the sections were incubated in secondary antibody diluted in blocking buffer for 30 min, washed again twice for 5 min in PBS and then incubated in Streptavidin/HRP diluted in blocking buffer for 30 min. After two washes in PBS for 5 min, the slides were visualized using 1 drop DAB Chromogen per mL substrate buffer (DAKO) for 5 min. In the final steps of both protocols, the sections were washed in MQ water for 5 min, counterstained using 0.5% methyl green (Sigma) in 0.1M Sodium Acetate Buffer for 2 min, washed twice for 3 min in MQ water, dipped 10 times in 96% ethanol, submerged in xylene and mounted

with Distyrene Plasticizer Xylene (DPX, Millipore Sigma). Images were captured using a Leica DFC490 digital camera, a Leica DMR microscope, Leica application suite software and Axio Scan Z.1.

2.4 Quantifying chromogen intensity using imageJ

To the human eye chromogen appears as varying degrees of coloration, thus the more antigen-chromogen is present, the darker the area appears. However, darker areas have lower intensity values resulting in an inverse correlation between the amount of antigen and its numerical value. Since the maximum intensity value of an RGB image analysed in ImageJ is 250, is possible to subtract the intensity of a stained region of interest (ROI) from 250, thereby deriving a reciprocal intensity that is directly proportional to the amount of antigen-chromogen present in the analysed sample (Nguyen et al., 2013). Considering that, this method was used on the immunostained pig paraffin sections to quantify the investigated marker expression. Using imageJ elliptical selection and "Measure" function present under the "Analyze" menu, in every image, the related white area maximum point was measured. Furthermore, the markers were expressed to varying degrees in tissues and subcellular localization: No stain (counterstain methyl green), weak stain (light brown), moderate stain (brown) and dark stain (dark brown). These four intensity

degrees for every marker were detected, to then calculate the average between these collected values, resulting in the average intensity of the expressed marker in the analysed image. The reciprocal intensity measurements was calculated and then ranges of reciprocal intensity values were set. Thereafter these ranges were converted in integer numbers, used to label the marker expression intensities in a heatmap (**Figure 19**) (**Table 2**) which was made using R studio software.

Reciprocal intensity value	**Corresponding integer number**	**Corresponding heatmap label**
0 - 50	0	No expression
50 - 120	2	Weak expression
120 - 170	4	Normal expression
170 - 250	6	Strong expression

Table 2. Reciprocal intensity values ranges with their corresponding integer number and heatmap label.

3 Results

The following results primarily report the morphological changing of developing pig heart at the developmental stages ranging from E15-E24 in which first heartbeat in the pig embryo is described (Hyttel, 2010; Trujano and

Wrathall, 1985). Therefore, the stages E15, E16, E18, E20, E22 and E24 were selected for this study, while the E70 pig heart was added to represent later stage of gestation (pig gestation is around 114 days) corresponding with developing human heart later stages. Secondly, these findings describe, in pig heart development, the investigated marker expression to identify different cell populations: cardiac mesoderm cells (PDGFR-α, FLK1), CPCs (ISL1, NKX2.5) and mature cardiomyocytes (CTNT, CX43, MYHC-B).

3.1 Anatomical characterization of the developing pig heart

Performing HE staining on the paraffin sections obtained from the selected pig embryos the anatomical aspects of the developing pig heart at the investigated embryonic days were evaluated (**Figure 12A**).

Figure 12B shows that at the stages E15 and E16, the cardiac tube is still smooth-walled but already segmented with dilatations, separated by indentations. In posterior-anterior position the expanded portions are the SV and the AT, which aren't enclosed within the pericardial cavity yet, the VE, the BC and the TA. Later the u-shaped cardiac tube is clearly visible at the stage E18 where it's possible to identify the beginning of trabeculation in the VE and AT, which is almost positioned above the VE, meaning the AT has entered the

pericardial cavity. Endocardial cushions (ECC) and conu-truncal cushions were apparent at the stage E20 meaning that Atrioventricular Canal (AVC) and the aorticopulmonary septum (AOS) are forming. The AVC will divide the future both left and right AT from the future either left and right VE, while the AOS will separate outlets from the primitive right and left VE into the ventral aorta and pulmonary trunk. At stage E22 the expansion of the primitive right AT toward the TA is evident. The valves in the AVC and the semilunar valves in the outflow tract (OFT) have also formed. Moreover, at E20 and E22 the developed trabeculation in the VE and BC myocardium can be identified. Another important observation is that at E24 the atrium has now surrounded the OFT.

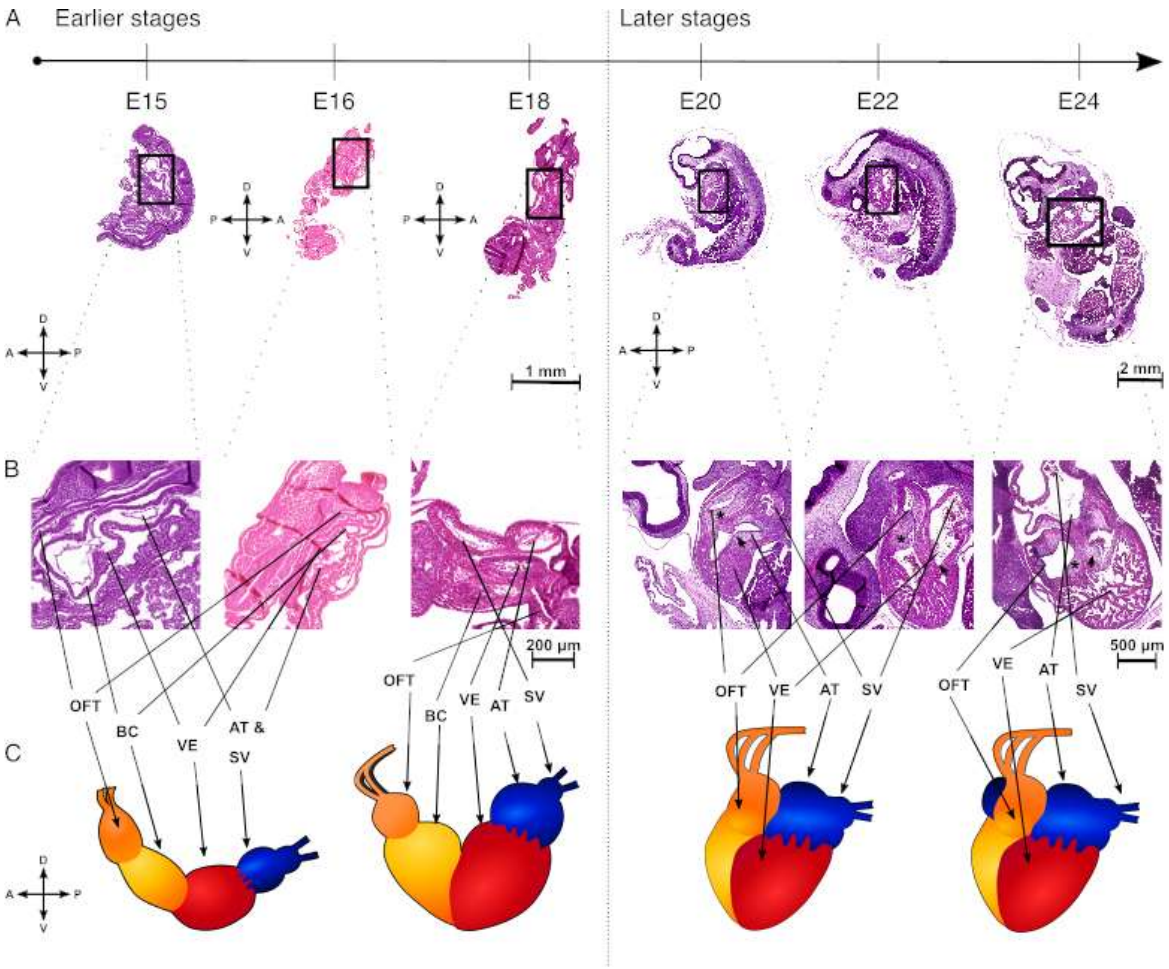

Figure 12. HE stained Pig embryos paraffin sections and their heart morphological changes at specific developmental stages. (A) HE stained Pig embryos sagittal sections viewed by left side (except E16 by right side). Orientation arrows: A=Anterior, P=Posterior, V=Ventral D=Dorsal. (B) Detailed magnification of developing pig heart at listed developmental stages (dashed lines). (C) Draw of pig developing heart at specific developmental stages. Abbreviations: AT=Atrium, BC=Bulbus cordis, SV=Sinus venosus, VE=Ventricle, OFT=Outflow tract, ECC=Endocardial cushions, *=conu-truncal cushions, little black arrow=endocardial cushions.

After the anatomical analysis pig embryonic days were compared with mouse and human known developing heart stages as following (D. Végh et al., 2016): pig E15/E16 linear tube corresponds to mouse E8 and human 3 gestation week, pig E18 corresponds to mouse E9.5 and human 3/4 gestation week, pig E20/E22 correspond to mouse E10.5 and human 4/5 gestation week, pig E24

corresponds to mouse E11.5 and human 5/6 gestation week (**Figure 13**). This temporal window relationship was used to compare markers expression among the three models as next described.

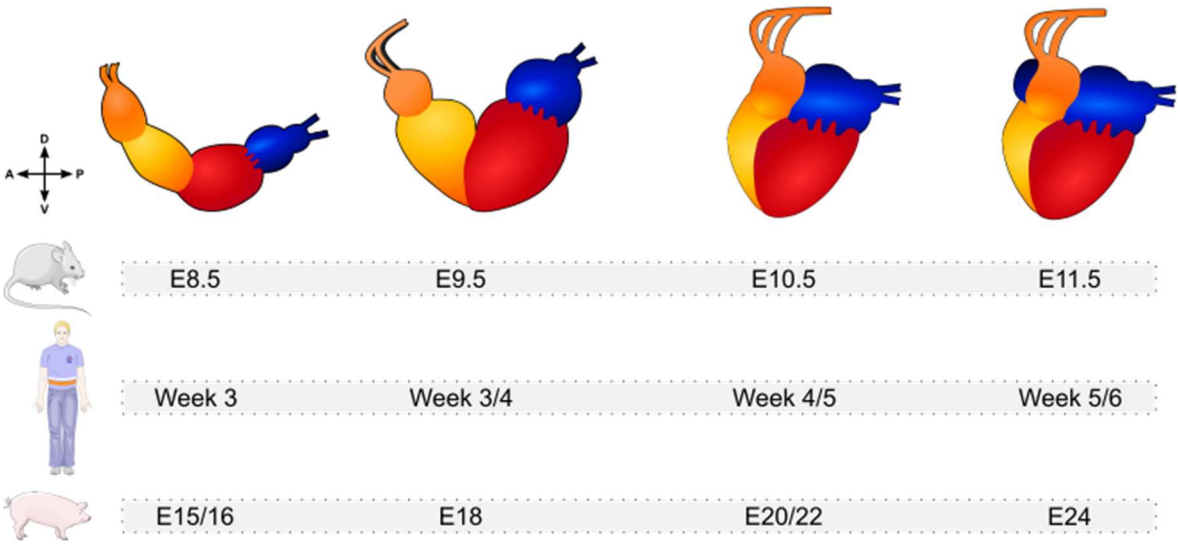

Figure 13. Heart developmental stages comparison between Mouse, Human and Pig. Orientation arrows: A=Anterior, P=Posterior, V=Ventral D=Dorsal.

3.2 Expression pattern of cardiac mesoderm cell markers in pig

FLK1 and PDGFR-α were investigated as cardiac mesoderm markers in developing pig heart, unfortunately the two PDGFR-α antibodies (Santa Cruz sc-398206, Abcam ab124392) used in the experiments did not work, thus only FLK1 results were obtained. As shown in **Figure 14**, FLK1 expression levels are moderate at early developmental stages in all cardiac tissues, mostly at E15

and E18, while it is downregulated in the myocardium and confined in endocardium and epicardium at later stages E20, E22 and E24, where it is weakly detected. Despite it has been evaluated as “No expression” by image J reciprocal intensity detection, low expression was observed by eye at E70 in myocardium and capillary vessels. At early stages E15, E16 and E18 FLK1 is located on the cell membrane and in cytoplasm, whereas at later stages its presence in the cytoplasm declines.

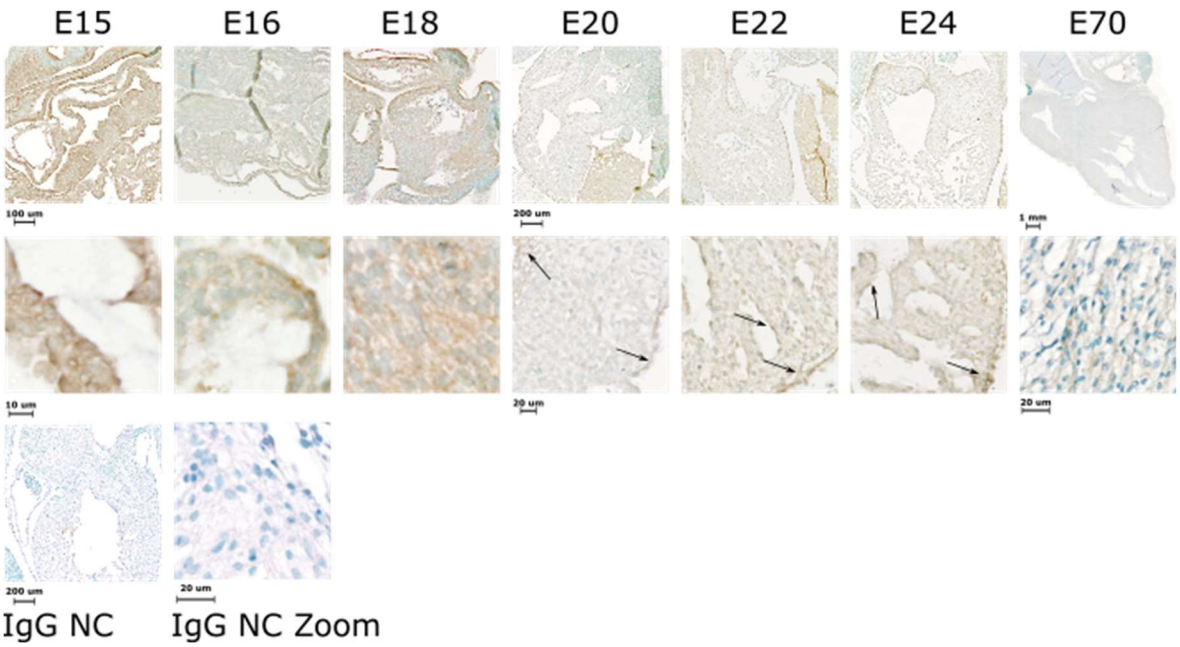

Figure 14. Flk1 expression in pig heart at developmental stages E15, E16, E18, E20, E22, E24 and E70. Black arrows indicate endocardium and epicardium. NC= Negative control.

3.3 Expression pattern of cardiac progenitor cell markers in pig

In pig embryos, ISL1 marker was observed present in all the investigated stages, expressed in all cardiac tissues and all the heart aspects. In myocardium and epicardium it was, interestingly, mainly located in cytoplasm, while it was both nuclear and cytoplasmic in the endocardium. The ISL1 detection is "normal" at E15, E16 and E18, to then increase at E20, E22 and E24. At E70, ISL1 was apparent in endocardium, myocardium, epicardium and aorta endothelial cells (**Figure 15**).

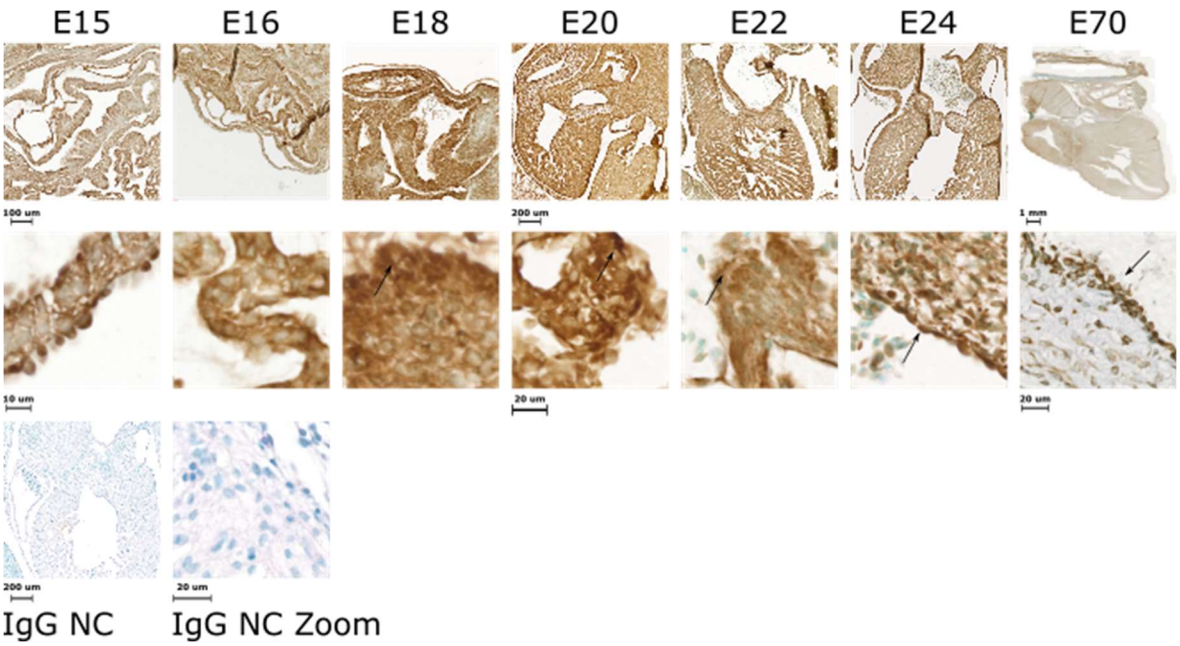

Figure 15. ISL1 expression in pig heart at developmental stages E15, E16, E18, E20, E22, E24 and E70. Black arrows indicate endocardium at E18, E20, E22, E24 and aorta endothelium at E70. NC= Negative control.

Regarding NKX2.5 expression in pig paraffin sections, it was detected at all investigated heart developmental stages except E15, moreover it was found cytoplasmic at E16 and E70, while it was located cytoplasmic but mostly nuclear in the other stages. Just as ISL1, NKX2.5 was evident in all cardiac tissues and all heart developing anatomical parts, showing a peak of expression at E18 and E22 (**Figure 16**).

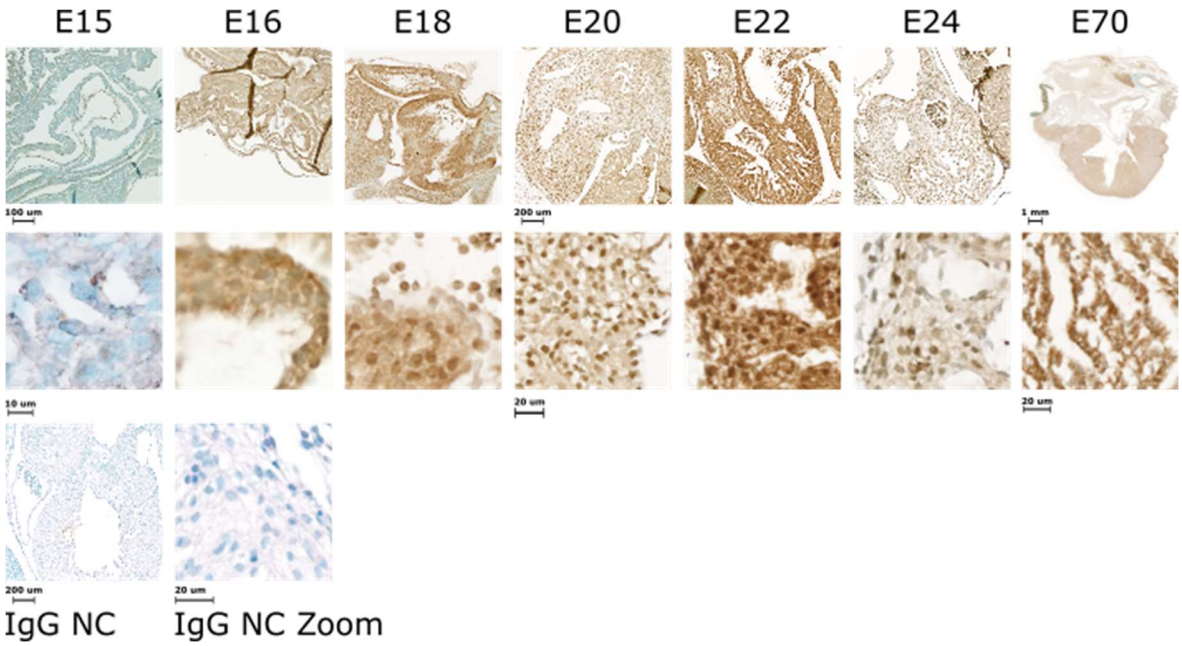

Figure 16. NKX2.5 expression in pig heart at developmental stages E15, E16, E18, E20, E22, E24 and E70. NC= Negative control.

3.4 Expression pattern of cardiomyocyte markers in pig

CTNT was located in the cytoplasm of the cardiac linear tube at E16 and in looped heart myocardial cells at E18, as well as in VE, AT and OFT

myocardium at E20, E22 and E24. Moreover CTNT expression in the cytoplasm persists at later stage E70 in the AT and VE myocardium. Peak of CTNT expression was observed at E18 and E22 (**Figure 17**).

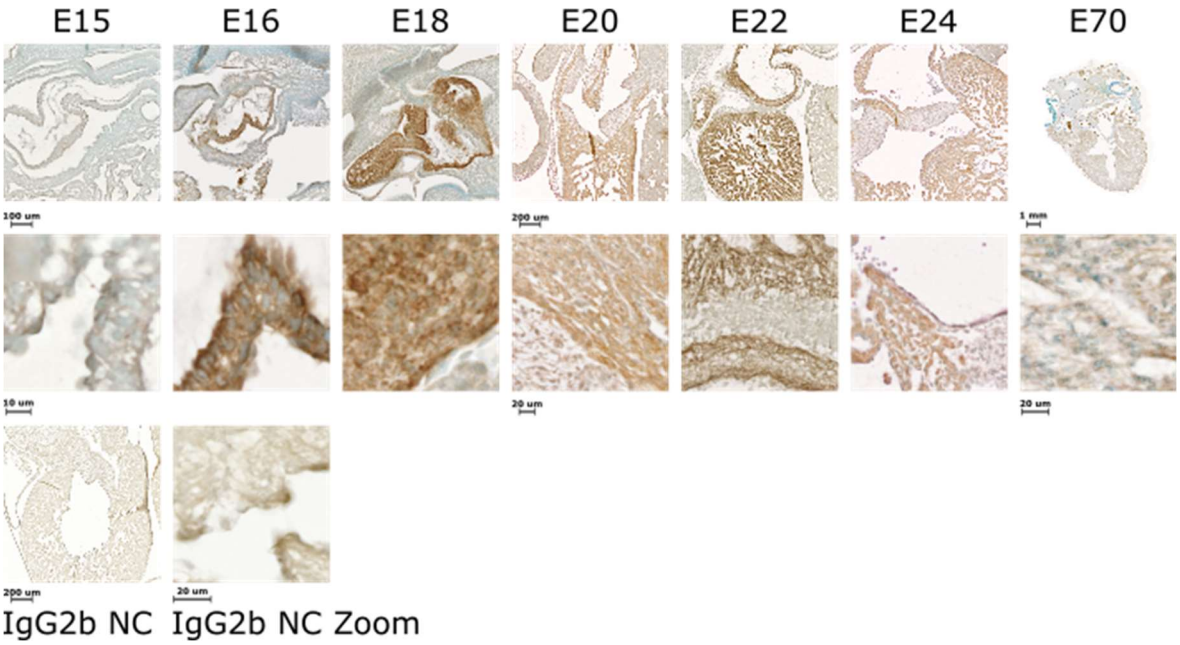

Figure 17. CTNT expression in pig heart at developmental stages E15, E16, E18, E20, E22, E24 and E70. NC= Negative control.

As shown in **Figure 18** and **Figure 20,** in pig embryos, MYHC-β is expressed at E16 and E18 in the cytoplasm of myocardial cells, in all cardiac tube and looping heart compartments, while it shows a peak of expression at E20 that persists at E22 and E24, which are the same stages where the marker gradually decrease its in the AT to be restricted in the VE and OFT. MYHC-β highlights its limitation in the VE at E70 (**Figure 18**).

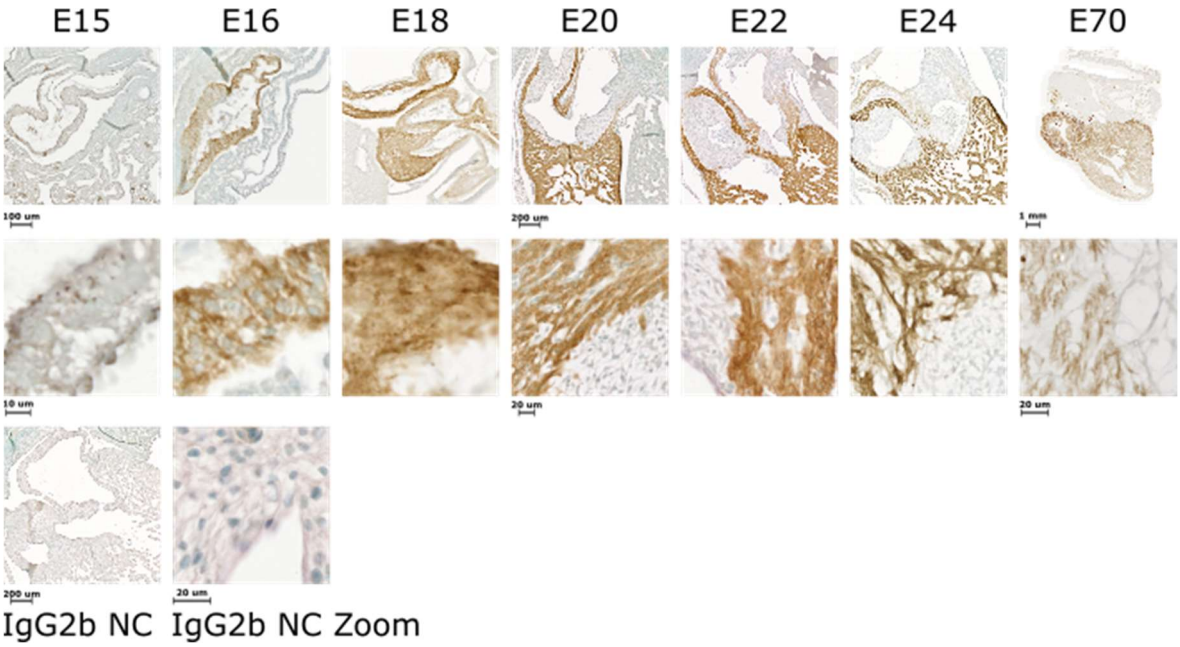

Figure 18. MYHC-β expression in pig heart at developmental stages E15, E16, E18, E20, E22, E24 and E70. NC= Negative control.

In developing pig heart, CX43 is expressed weakly at E15, E16 and E18 to then increase its intensity at later developmental stages E20, E22 and with a peak of expression at E24 (**Figure 19**). Of note, at E20 CX43 is expressed on the membrane in endocardium, myocardium and less in epicardium, as well as in ECC and conotruncal cushions. However it becomes more and more confined in myocardium in the other later developmental stages.

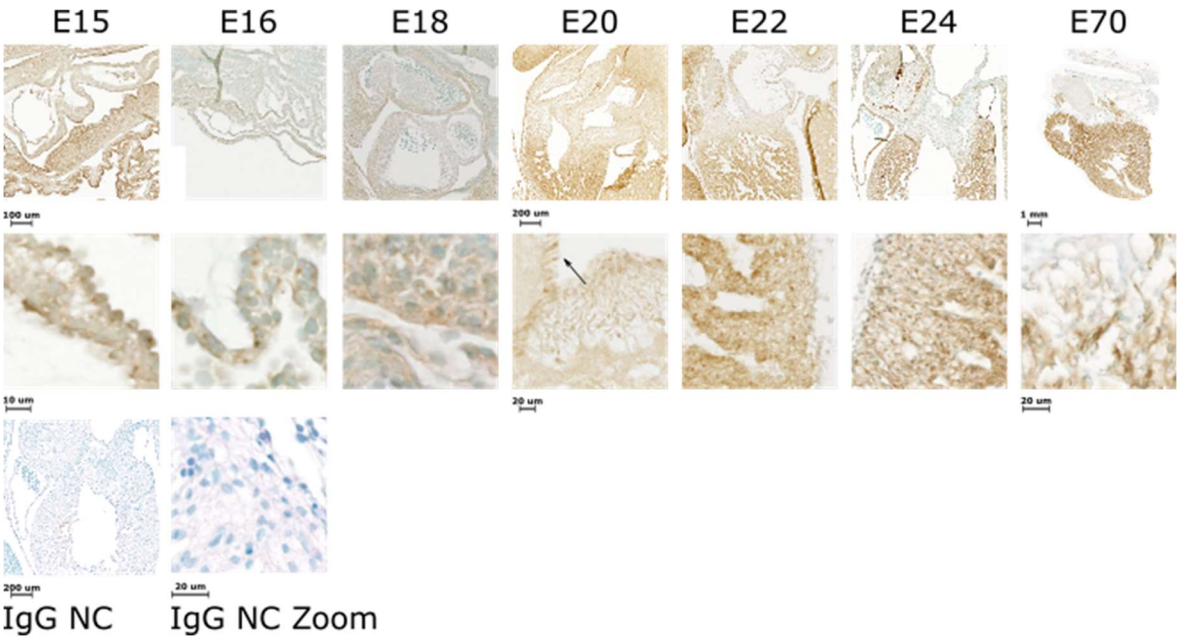

Figure 19. CX43 expression in pig heart at developmental stages E15, E16, E18, E20, E22, E24 and E70. Black arrows indicate ECC. NC= Negative control.

Figure 20. Heatmap representing the investigated markers expression in developing pig heart at developmental stages E15, E16, E18, E20, E22, E24 and E70, with their tissue and subcellular location. End = Endocardium, Myo = Myocardium, Epi = Epicardium.

4 Discussion

The anatomical analysis results allowed to identify important morphological transformations in developing pig heart and to associate the timing of key events with corresponding human and mouse heart developmental stages in order to compare, among these mammals models, the investigated marker expression pattern, which in some case have proved more similarities between human and pig models than between human and mouse models.

4.1 Developing pig heart anatomical evaluation

Pig embryonic heart is a linear segmented tube at E15/E16 and undergoes a loop at E18, to then show ECC and semilunar valves appearance at E20 and E22, while it manifests the AT surrounding the OFT at E24. These key events made possible to find and compare the same developing heart temporal window in mice and humans (**Figure 13**). This anatomical evaluation corresponds with previous studies reporting the heart starts to beat as early as the linear segmented heart tube stage (Trujano and Wrathall, 1985, Christoffels et al., 2010), here represented by developing pig heart at E15 where circulating blood cells are visible (**Figure 12A,B,C**).

4.2 Comparative expression pattern of cardiac mesoderm cell markers in pig, human and mice

The results for FLK1 confirm data reported in the scientific literature from mouse and human studies (Yamaguchi et al., 1993; Vieira et al., 2010; Partanen et al., 1999, Wagner et al., 2005) and further confirms FLK1 expression in the epicardium at later stages (Partanen et al., 1999, Wagner et al., 2005), in contrast to literature declaring FLK1 loss in epicardial cell population (Brade et al., 2013). Interestingly, FLK1 membrane and cytoplasmic location at early stages E15, E16 and E18, matches with literature reporting FLK1 to be cytoplasmic during its translocation in the nucleus following vascular endothelial growth factor (VEGF) or hypoxic stimulation (Blazquez et al., 2006). Additionally, decreasing levels of FLK1 suggests stronger expression of this marker may precede the investigated stages. In sum, FLK1 was validated to be a good cardiac mesoderm marker for pig embryos.

4.3 Comparative expression pattern of cardiac progenitor cell markers in pig, human and mice

Notably ISL1's cytoplasmic location detected in myocardium and epicardium, was speculated to might be an inactive form of the transcription factor. In mice,

ISL1 is expressed at early stages of development and is rapidly lost (Iancu et al., 2015), however these data are in contrast with ISL1 presence in the E70 pig heart, where ISL1 was still expressed in the cytoplasm within the myocardium, while it was nuclear in the aorta endothelium (OFT). These results are similar to those reported in the human where ISL1 persists at later stages of cardiogenesis (Bu et al., 2009) (**Figure 14**). This indicates that ISL1 expression in the pig is more related to the human than the mouse.

In contrast to the mouse model and similarly to human model (Iancu et al., 2015), NKX2.5 is first expressed after ISL1 (from E16) and overlaps temporally with CTNT and FLK1. In addition, the detection of NKX2.5 and ISL1 in all cardiac tissues and structures, strengthen the scientific papers reassessing as markers to identify CPCs composing both FHF and SHF (Ma et al., 2008).

4.4 Comparative expression pattern of cardiomyocyte markers in pig, human and mice

CTNT is the most commonly used marker to identify cardiomyocytes (Forough et al., 2011). CTNT expression was detected as early as E7.5 in mice (Wang et al., 2001), which is, in comparison, before than CTNT first appearance in the

pig at E16, to then persists, in accordance with human data (Cui et al., 2019), until later stage E70 (**Figure 16**). Interestingly CTNT shows the same expression pattern as NKX2.5 (**Figure15, Figure 16 and Figure 19**) suggesting that CTNT expression is regulated by the transcription factor NKX2.5 as already reported in scientific literature (Wang et al., 2001). Since CTNT is expressed only in pig heart myocardium, it is confirmed to be a good cardiomyocytes marker which allowed to recognise and localize the cardiac structures during the development.

The results for MYHC-β show the same mouse and human expression pattern before the birth (Morkin, 2000), however this marker is known to be expressed in human VE from the fetus to adult, in contrast to mouse model where, in the VE, MYHC-β is completely replaced by MYHC-α postnatally (Morkin, 2000). As well as CTNT, MYHC-β myocardial characterization, suggests it as good cardiomyocyte marker which allocate the cardiac structures at early stages and indicate the VE at later stages.

The expression of CX43 in the endocardium, ECC and conotruncal cushions doesn't correspond to data reported from mice and human models, where are cited other connexin proteins to contribute to such structures (Giovannone et al., 2012, Kaba et al., 2001). The collected data in developing pig heart, describing CX43 expressed in the AT at E70, are more similar with mouse

model (Giovannone et al., 2012) since in human fetal heart CX43 is very low detectable in the AT (Kaba et al., 2001).

5 Conclusions and future perspectives

With this study, for the first time, was characterised the anatomical development of the pig heart at specific embryonic developmental days and associated the timing of key events with corresponding human and mouse heart developmental stages. Furthermore, specific markers which identify cell populations involved in cardiac development, which has not been done in the pig model before, were investigated. The results are, on one hand in accordance with the mouse model, on the other hand, the pig model appeared more similar to human model than mouse, like for ISL1 expression after NKX2.5 and its presence at later stages of development, as well as NKX2.5 expression overlapped with FLK1 and CTNT. In addition, the obtained results confirm previous data reporting FLK1 expressed in the epicardium; however contrary to mouse model CTNT was detected after the corresponding stage in mouse, but is not possible to compare this aspect with human model since there aren't available data for this marker at so early stages. Importantly, this study strengthens previous results which report NKX2.5 and ISL1 expressed in both

heart fields and outline CTNT and MYHC-β as good cardiomyocyte markers, which allow to recognise the cardiac structures during developmental stages.

Taken together, these findings shed light on an alternative model for human heart development, namely the pig. However, these data needs to be supported by more specific quantitative marker expression analysis such as Real-time PCR or western blot and in conclusion, MYHC-β should be further investigated in post-natal and adult pig heart to evaluate its expression in the VE or its eventual replacement by MYHC-α. Such analysis would further affirm the pig to be a more similar model to the human than the mouse for studying heart development.

BIBLIOGRAPHY

- Blazquez Cristina, Nathan Cook, Kingsley Micklem, Adrian L Harris, Kevin C Gatter, Francesco Pezzella. 2006. Phosphorylated KDR can be located in the nucleus of neoplastic cells. Cell Research (2006) 16:93-98. doi:10.1038/sj.cr.7310012.

- Brade Thomas, Luna S. Pane, Alessandra Moretti, Kenneth R. Chien, and Karl-Ludwig Laugwitz. 2013. Embryonic Heart Progenitors and Cardiogenesis.
- Bu Lei, Xin Jiang, Silvia Martin-Puig, Leslie Caron, Shenjun Zhu, Ying Shao, Drucilla J. Roberts, Paul L. Huang, Ibrahim J. Domian, & Kenneth R. Chien. 2009. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages.
- Calderon Damelys, Evan Bardot, Nicole Dubois. 2016. Probing early heart development to instruct stem cell differentiation strategies.
- Christoffels M. Vincent, Gertien J. Smits, Andreas Kispert, Antoon F. M. Moorman. 2010. Development of the Pacemaker Tissues of the Heart. Circ Res. 2010;106:240-254.
- Clowes Christopher, Michael G.S. Boylan, Liam A. Ridge, Emma Barnes, Jayne A. Wright, and Kathryn E. Hentges. 2014. The Functional Diversity of Essential Genes Required for Mammalian Cardiac Development.
- Cui Yueli, Yuxuan Zheng, Xixi Liu, Lu Wen, Jie Qiao, Fuchou Tang. 2019.Single-Cell Transcriptome Analysis Maps the Developmental Track of the Human Heart. Cell Reports 26, 1934–1950 February 12, 2019 ª 2019 The Author(s). https://doi.org/10.1016/j.celrep.2019.01.079.

- Forough Reza, PhD, Catherine Scarcello, BA, Matthew Perkins, BA. 2011. Cardiac Biomarkers: a Focus on Cardiac Regeneration.
- Giovannone Steven, Benjamin F. Remo, and Glenn I. Fishman, FHRS, Leon H. 2012. Channeling Diversity: Gap Junction Expression. Heart Rhythm. 2012 July; 9(7): 1159–1162. doi:10.1016/j.hrthm.2011.11.040.
- Hyttel, P., 2010. Essentials of domestic animal embryology. Saunders/Elsevier.
- IANCU CRISTIAN BOGDAN, DANIELA IANCU, IRINA RENŢEA, SORIN HOSTIUC, DAN DERMENGIU, MUGUREL CONSTANTIN RUSU. 2015. Molecular signatures of cardiac stem cells.
- Ishitobi Hiroyuki, Asami Wakamatsu, Fang Liu, Takuya Azami, Michito Hamada, Ken Matsumoto, Hiroshi Kataoka, Makoto Kobayashi, Kyunghee Choi, Shin-ichi Nishikawa, Satoru Takahashi and Masatsugu Ema. 2011. Molecular basis for Flk1 expression in hemato-cardiovascular progenitors in the mouse.
- Jensen Bjarke, Tobias Wang, Vincent M. Christoffels, Antoon F.M. Moorman, 2013. Evolution and development of the building plan of the vertebrate heart.
- Kaba A. Riyaz, Steven R. Coppen, Emmanuel Dupont, Jeremy N. Skepper, Suzy Elneil, Marcus P. Haw, John R. Pepper, Magdi H. Yacoub, Stephen Rothery & Nicholas J. Severs (2001) Comparison of Connexin 43, 40 and 45 Expression Patterns in the Developing Human and Mouse Hearts, Cell Communication & Adhesion, 8:4-6, 339-343, DOI: 10.3109/15419060109080750.
- Kataoka Hiroshi, Nobuyuki Takakura, Satomi Nishikawa, Kunihiro Tsuchida, Hiroaki Kodama, Takahiro Kunisada, Werner Risau, Toru Kita, Shin-Ichi Nishikawa. 1997. Expressions of PDGF receptor alpha, c-Kit and Flk1 genes

clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells.

- Kobayashi Toshihiro, Haixin Zhang, Walfred W. C. Tang, Naoko Irie, Sarah Withey, Doris Klisch, Anastasiya Sybirna, Sabine Dietmann, David A. Contreras, Robert Webb, Cinzia Allegrucci, Ramiro Alberio & M. Azim Surani. 2017. Principles of early human development and germ cell program from conserved model systems.
- Ma Qing, Bin Zhou, William T. Pu. 2008. Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity.
- MORKIN EUGENE. MICROSCOPY RESEARCH AND TECHNIQUE 50:522–531 (2000). Control of Cardiac Myosin Heavy Chain Gene Expression.
- Moses A. Kelvin, Franco DeMayo, Renee M. Braun, James L. Reecy, and Robert J. Schwartz*. 2001. Embryonic Expression of an Nkx2-5/Cre Gene Using ROSA26 Reporter Mice. DOI 10.1002/gene.10022.
- Nguyen DH, Zhou T, Shu J, and Mao JH. 2013. Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity. Cancer InCytes: http://www.cancerincytes.org/currentissue/letterfromtheeditorinchief.html#!quantifying-chromogenintensity-in-immunohistochemistry- /c1vds. http://www.ihcworld.com/_books/Nguyen_Reciprocal%20Intensity.pdf.
- Paige L. Sharon, Karolina Plonowska, Adele Xu, Sean M. Wu. 2015. Molecular Regulation of Cardiomyocyte Differentiation. DOI:10.1161/CIRCRESAHA.116.302752.

- Partanen A. Taina, MD; Taija Makinen, MSc; Johanna Arola, MD, PhD; Toshio Suda, MD, PhD; Herbert A. Weich, MD, PhD; Kari Alitalo, MD, PhD. 1999. Endothelial Growth Factor Receptors in Human Fetal Heart.
- Qian C, Wong CWY, Wu Z, He Q, Xia H, Tam PKH, et al. (2017) Stage specific requirement of platelet-derived growth factor receptor-α in embryonic development. PLoS ONE 12(9): e0184473. https://doi.org/10.1371/journal.pone.0184473.
- Robert G. Kelly. 2016. How Mesp1 makes a move.
- RUANGVORAVAT P. CHRISTINE AND CECILIA W. LO. 1992.Connexin 43 Expression in the Mouse Embryo. Localization of Transcripts Within Developmentally Significant Domains.
- Tosic Jelena, Gwang-Jin Kim, Mihael Pavlovic, Chiara M. Schröder, Sophie-Luise Mersiowsky, Margareta Barg, Alexis Hofherr, Simone Probst, Michael Köttgen, Lutz Hein and Sebastian J. Arnold. 2019. Eomes and Brachyury control pluripotency exit and germ-layer segregation by changing the chromatin state. https://doi.org/10.1038/s41556-019-0423-1.
- Trujano, M., Wrathall, A.E., 1985. Observations on the development in vitro of early (13-day) and later (15-day) porcine embryos. Br. Vet. J. 141, 378–387. https://doi.org/10.1016/0007-1935(85)90088-0.
- Végh M. D. Anna, Sjoerd N. Duim, Anke M. Smits , Robert E. Poelmann, Arend D. J. ten Harkel, Marco C. DeRuiter, Marie José Goumans and Monique R. M. Jongbloed. 2016. Part and Parcel of the Cardiac Autonomic Nerve System: Unravelling Its Cellular Building Blocks during Development.
- Vieira Miguel Joaquim , Christiana Ruhrberg and Quenten Schwarz. 2010. VEGF receptor signaling in vertebrate development.

- Wagner Nicole, Kay-Dietrich Wagner, Heinz Theres, Christoph Englert, Andreas Schedl,and Holger Scholz. 2005. Coronary vessel development requires activation of the TrkB neurotrophin receptor by the Wilms' transcription factor Wt1.
- WANG QIN, REBECCA S. REITER, QI-QUAN HUANG, JIAN-PING JIN, AND JIM JUNG-CHING LIN1. 2001.Comparative Studies on the Expression Patterns of Three Troponin T Genes During Mouse Development. THE ANATOMICAL RECORD 263:72–84 (2001) © 2001.
- Xiong Haiqing & Aibin He. 2020. Single-Cell Transcriptomic Analysis of Cardiac Progenitor Differentiation.
- Yamaguchi P. Terry , Daniel J. Dumont, Ronald A. Conlon, Martin L. Breitman and Janet Rossant. 1993. flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors.
- Zhuang Shaowei, Qingquan Zhang1, Tao Zhuang, Sylvia M. Evans, Xingqun Liang, and Yunfu Sun. 2013. Expression of Isl1 during mouse development.

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