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A comparative assessment of marker expression between cardiomyocyte differentiation of human iPSCs and the developing pig heart

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Abstract

The course of differentiation of pluripotent stem cells into cardiomyocytes and the intermediate cell types are characterized using molecular markers for different stages of development. These markers have been selected primarily from studies in the mouse and from a limited number of human studies. However, it is not clear how well mouse cardiogenesis compares with human cardiogenesis at the molecular level. We tackle this issue by analyzing and comparing the expression of common cardiomyogenesis markers (PDGFR- α , FLK1, ISL1, NKX2.5, CTNT, CX43 and MYHC-B) in the developing pig heart at embryonic day (E)15, E16, E18, E20, E22 and E24 and in differentiating cardiomyocytes from human induced pluripotent stem cells (hiPSC). We found that porcine expression of the mesoderm marker FLK1 and the cardiac progenitor marker ISL1 was in line with our differentiating hiPSC and reported murine expression. The cardiac lineage marker NKX2.5 was expressed at almost all stages in the pig and human iPSC, with an earlier onset in the hiPSC compared to reported murine expression. Markers of immature cardiomyocytes, CTNT and MYHC-B were consistently expressed throughout E16 – E70 in the pig, which is comparable with mouse development, whereas the markers increased over time in the hiPSC. However, the commonly used mature cardiomyocyte marker, CX43, should be used with caution, as it was also expressed in the pig mesoderm, as well as hiPSC immature cardiomyocytes, whilst this has not been reported in mice. Based on our observations in the various species, we suggest to use FLK1/PDGFR- α for identifying cardiac mesoderm, ISL1/NKX2.5 for cardiac progenitors and CTNT/MYHC-B for immature cardiomyocytes. Further, CTNT+/ISL1+ could mark immature cardiomyocytes and CTNT+/ISL1- mature cardiomyocytes. CX43 should be used together with sarcomeric proteins. This knowledge may help improving differentiation of hiPSC into more *in vivo* like cardiac tissue in the future.

Keywords: Heart development, embryoid body, cardiac marker, pig, induced pluripotent stem cell, cardiomyocyte differentiation

1. Introduction

Replicating heart development *in vitro* using pluripotent stem cells (PSCs) is dependent on a series of developmental changes in a short time frame that is verified using well-established cardiomyocyte markers. As PSCs are guided through cardiomyogenesis, they undergo developmental changes similar to those in the developing embryo, including mesoderm formation, marked by expression of T Brachyury (BRACHYURY) and cardiac mesoderm marked by posterior BHLH transcription factor (MESP1), Fetal liver kinase 1 (FLK1) and Platelet-Derived Growth Factor Receptor, Alpha Polypeptide (PDGFR- α). These cells then form cardiac progenitors that express insulin gene enhancer protein (ISL1), the NK2 homeobox 5 (NKX2.5), GATA binding protein 4 (GATA4) and T-Box transcription factor 5 (TBX5), before they differentiate into immature cardiomyocytes marked by cardiac troponin T (CTNT), Myosin Heavy chain 7 (MYHC-B) and other cell surface markers [1]. Maturation of cardiomyocytes takes several weeks and is more complex to assess, as not only markers of gap junctions such as gap junction protein Connexin43 (CX43) are used to characterize maturation [2–4], but also electrophysiological methods, calcium profiles, myofibril organization and sarcomere striation [1]. These gene markers are generally accepted in the field and have been identified from mouse (and to a much lesser extent from human) cardiac development studies and are applied to human stem cell differentiation studies to identify the different stages of cardiac differentiation [1,5–10]. To our knowledge, only three studies have investigated gene expression in human fetal hearts [11–13] and comparisons to mouse heart development indicate that differences exist. For example, MYHC-B is upregulated in human ventricular cardiomyocytes while it is downregulated in these cells in mice [14]. Further, different mesoderm populations exist in the human which form for e.g. CD235A⁺ cardiomyocytes of the left ventricle, which have not been identified in the developing mouse heart [15]. Another study on human PSC-derived cardiomyocyte-like cells describes a novel marker for cardiac progenitor cells, LGR5, that is unique to human cardiac progenitor cells [11]. It is also not clear if the markers used for cardiac differentiation studies are especially accurate for human tissues. Unfortunately, investigation of human heart development remains limited due to the rarity of available tissue to researchers.

Identification of alternative animal models of heart development, which better replicate the molecular mechanisms driving human cardiac development would be an advantage for the field. The pig is a strong candidate, since pig embryos develop as bilaminar embryonic discs (flat disc) just like human embryos [16].

Developing pig embryos and fetuses are easily obtained through pig production farms and are a good alternative to using non-human primate embryos/fetuses for studying development [17–21]. A detailed molecular characterization of early pig heart development is absent from the literature. Only limited knowledge is available on the prenatal developing heart [22,23] and one study was performed on the postnatal developing heart [24]. Molecular profiling has recently been performed on the adult pig heart, which shows clear commonalities with the mouse heart, relating to metabolic processes such as carbohydrate metabolism. Unique proteins were also expressed in the pig heart although not clearly depicted in the article [25]. Here, we provide the first molecular studies of cardiogenesis in the pig and cross-compare it to an established human PSC-based cardiac differentiation protocol [26], which results in almost 100 % contracting cardiac spheres after seven days.

The aim of this study was to determine whether commonly used markers of early heart development were aligned between the pig and differentiating human iPSC cardiomyocytes and to assess whether there were differences to published mouse studies. We provide detailed anatomical and molecular insight into the developing pig heart, with focus on the most commonly used markers PDGFR- α , FLK1 (cardiac mesoderm), ISL1, NKX2.5, (cardiac progenitors) MYHC-B, CTNT (immature cardiomyocytes) and CX43 (mature cardiomyocytes) in an attempt to assess whether refinement of the use of molecular markers used in human cardiac differentiation studies is needed.

2. Materials and methods

2.1 Pigs embryos and fetal pig heart

Pig embryos were obtained at day embryonic day (E)15, E16, E18, E20, E22 and E24 of gestation (three embryos per day) following insemination of Landrace/Yorkshire sows (twice within 24 h) 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. A pig heart from E70 was also dissected from a fetus. The embryos and heart were fixed in 4 % paraformaldehyde (PFA) in PBS at 4 °C overnight and stored until use in PBS at 4 °C containing 0.02 % sodium azide.

2.2 Tissue preparation and Haematoxylin and Eosin staining

The embryos and heart were paraffin embedded in a tissue processor (Thermo Fisher Scientific, Waltham, USA), 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, Radnor, USA). From the paraffin blocks sagittal sections (5 µm) were cut using a microtome (SM2000R, Leica, Wetzlar, Germany). The sections were mounted onto glass slides and stored at 4 °C. 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, Thermo Fisher Scientific, PA5-49431), ISL1 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). The following negative controls were used: Negative Mouse IgG1 (1:40, DAKO, x0931), Normal Rabbit IgG (1:2000, DAKO, x0936), Negative Mouse IgG2b (1:40, DAKO, x0944). These antibodies were isolated from non-immunized animals and are well suited to identify non-specific binding to cell surface components. The following secondary antibodies were used: Secondary Antibody Biotinylated goat anti-rabbit (1:250, DAKO, E0432), Third Layer Streptavidin/HRP (1:500, DAKO, P0397, lot. 00032671, 0.83g/L). 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 times 5 min in boiling 0.01 M sodium citrate solution (PH 6, Merck Millipore, Burlington, USA). Sections were then washed in 4 °C 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. Again the sections were 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, Glostrup, Denmark) for 5 min. For the polyclonal rabbit antibody, sections were kept 5 min in PBS following rehydration and permeabilized with 0.1% TritonX-100 (Merck Millipore) 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 % H₂O₂ 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, Merck Millipore) 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 one 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-Aldrich, St. Louis, USA) in 0.1 M 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, Merck Millipore). Images were captured using a Leica DFC490 digital camera and a Leica DMR microscope and Leica application suite software.

2.6 hiPSC culture

For experiments with human cells, the hiPSC line Bioni010-C (Bioneer A/S, Hoersholm, Denmark) was used. Cells were cultured in mTeSR™1 serum-free medium (STEMCELL Technologies, Vancouver, Canada) in 6 cm cell culture dishes (Nunc™ Delta Surface, Thermo Fisher Scientific) coated with hESC-Qualified Matrigel (354277, Corning, Corning, USA). The medium was exchanged daily and cells were split weekly with EDTA. Briefly, medium was removed from the dish, the cells washed once with DPBS (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich) and 1 ml 0.02 % EDTA in DPBS added. The cells were incubated for 1-3 min and the EDTA was removed. The cells were harvested in small clumps in mTeSR™1 and transferred to a new plate according to the split ratio. The hiPSCs were incubated at 37 °C and 5 % CO₂.

2.7 Embroid body formation and cardiac differentiation

3D cultures of embryoid bodies (EBs) that mimic the early human blastocyst were generated from hiPSCs and subsequently differentiated into cardiomyocytes as described in [26]. For harvesting hiPSCs, medium was removed from the culture dish and 1 ml Gibco™ TrypLE™ Select (Thermo Fisher Scientific) added and

incubated for 1-2 min. TrypLE was removed and the cells detached from the plate by adding 10 ml mTeSR-ROCK (mTeSR1 with Y27632 dihydrochloride). A single cell suspension was generated by pipetting several times. The cells were counted and diluted to 5×10^4 cells/ml, of which 100 μ l per well were seeded into a 96-Well Polystyrene Conical Bottom MicroWell™ Plate (249952, Thermo Fisher Scientific). The plates were centrifuged at 500 g for 5 min and incubated at 37 °C and 5 % CO₂. 20 h after seeding, and thereafter daily, medium was exchanged by removing 80 μ l of medium and replaced with 80 μ l fresh medium according to this schedule: day 0 (20 hr after seeding): day 0 medium, day 1: TS medium, day 2: Wnt medium, day 3: TS medium, day 6: TS-medium (removal of 60 μ l per well and addition of 80 μ l per well on day 6). Media recipes are given in supplementary material.

2.8 Immunofluorescence of embryoid bodies

The EBs were transferred from a culture plate to a 15 ml centrifugation tube with a 1000 μ L pipette (wide orifice tips). The spheres were left undisturbed for 5 min to allow gravity precipitation and all but approximately 0.5 ml supernatant was removed. Subsequently the spheres were resuspended and transferred to a 0.5 ml tube. For fixation, excess medium was removed and the spheres were washed once in PBS and fixed in 500 μ l 4 % formaldehyde for 15 min at room temperature (RT). The formaldehyde was removed and the spheres washed three times in PBS and then stored in PBS at 4 °C. Next spheres were permeabilized for 1 h at RT using 0.1 % Triton-X (Merck Millipore) in PBS, washed 3 x in PBS and incubated in blocking buffer (5 % Normal donkey serum (NDS) and 1 % BSA in PBS) at RT for 2 h. Primary antibodies (see section 2.3 and SSEA3 rat (Biolegend 330302, 1:100), OCT4 goat (sc-8628, 1:100), SOX2 mouse (Millipore, SC1002, 1:1000)) diluted in blocking buffer were incubated with the EBs on an orbital shaker at 4 °C overnight using gentle rotation. EBs were washed in PBS for 10 min at RT, then incubated with secondary antibodies (Donkey anti-rabbit: ab150075 (AlexaFluor 647) Abcam (1:200), Donkey anti-mouse: ab150105 (AlexaFluor 488) Abcam (1:200), Donkey anti-goat: ab150131 (AlexaFluor 647) Abcam (1:200)) in blocking buffer for 1 h at RT in the dark and then washed again in PBS for 15 min at RT. For negative controls, D0 EBs were incubated without primary antibody and only with secondary antibodies. For DNA labelling, EBs were incubated in Hoechst solution 10 μ g/ml (Merck Millipore) for 10 min in the dark. In the end, EBs were washed in PBS in the dark and immediately prepared for mounting, thus transferred to glass slides and mounted with fluorescent mounting solution (DAKO).

2.9 Chromogen intensity quantification

In order to create an overview of the expression data in the pig embryos, we estimated the expression levels and classified them into four categories, which were then displayed in a heat map. For this, reciprocal intensity of the chromogen stained immunohistochemistry sections was measured in order to quantify the amount of marker protein present at the respective stages of pig heart development following the method by [27]. For that, the images were opened in ImageJ and the area of interest selected with the elliptical selection tool followed by the 'measure function' to retrieve the white area maximum. This value was subtracted from 250, which is the maximum intensity value of white colour in a JPG image. The average reciprocal intensity for every marker and developmental stage was calculated after measuring every image from three embryos. For graphical illustration in a heat map, the ranges were converted into integer numbers according to Suppl. Table 2. With these values, a heat map was created using R studio.

3. Results

3.1 Anatomical characterization of pig heart development

There is little anatomical characterization of the developing prenatal pig heart, therefore, it was crucial to first determine the timing of key anatomical stages of cardiac development. We decided to select embryos from E15 – E24, since this is the range in which we presumed the heart begins to beat based on two reports [22,23] as well as a later stage embryo from E70 to identify expression at a later midway stage during gestation (gestation length in pig = 114 days) (Fig. 1A). At E15 and E16, the pig cardiac tube was still smooth-walled but clearly segmented into four dilatations, separated by indentations in a posterior-anterior orientation. The expanded parts identifiable were the sinus venosus (SV) and the atrium (AT) (not yet enclosed within the pericardial cavity), the ventricle (VE) and the bulbus cordis (BC) (Fig. 1A). Note, the truncus arteriosus is not visible in these sections, since it is located on the right lateral side of the embryo. No circulation of red blood cells could be observed at E15 or E16 of development. At E18, the cardiac tube was beginning to fold. It was u-shaped and trabeculation was observable within the VE and BC. The AT was positioned above the VE and enclosed in the pericardial cavity (Fig. 1A). We could also observe at this time point circulation of red blood cells through the heart cavities. At E20, endocardial cushions and conotruncal cushions appeared, forming the atrioventricular canal (AVC) and the aortic-pulmonary septum (Fig. 1A). At E22, the expansion of the primitive left AT toward the truncus arteriosus was evident, and it was possible to observe the formed valves in the AVC

and semilunar valves in the outflow tract (OFT). Furthermore, the formed trabeculation was visible in the VE myocardium wall, where the interventricular septum already appeared (Fig. 1A). At stage E24, the mitral valves were observable in the AVC, as well as the semilunar valves in the OFT, regulating the blood flow, and the atrium had surrounded the OFT (Fig. 1A). This anatomical description was important for defining the specific timing of key events during early pig heart development, which are summarized in Fig. 1B and compared to the same anatomical stages in mouse and human.

3.2 Our in vitro cardiomyocyte differentiation model

We applied an EB-based differentiation method to produce hiPSC-derived cardiomyocytes based on the use of growth factors from the ActivinA/Nodal, BMP4 and Wnt pathways, which yields beating cardiomyocytes after seven days [26]. The EBs on day (D)0 consisted of pluripotent cells as they expressed Oct3/4, Sox2 and SSEA4 (Suppl. Fig. 1). The differentiation efficiency was very high as almost all EBs beat rigorously on D7, as we showed before [26]. Since no maturation step was included, the cardiomyocytes on D7 represented immature cardiomyocytes, as present in the embryo. Our cardiomyocyte differentiation model was therefore in alignment with the developmental timeframe covered in our pig study, which corresponds to mouse E8.0 – E11.5 and human gestational day 21 – 42 (Fig. 1B).

3.3 Expression pattern of cardiac mesoderm and cardiac progenitor markers PDGFR- α , FLK1, ISL1 and NKX2.5 in the developing pig heart and differentiating human iPSC

PDGFR- α and FLK1 together are well-established markers for cardiac mesoderm [28,29]. We investigated only FLK1 expression in the developing pig heart, since two PDGFR- α antibodies (Santa Cruz sc-398206, Abcam ab124392) we tested in our pig tissues failed to work (likely due to species-specific differences in the antibody peptides). We found that FLK1 was expressed at the earliest time point studied, E15, in the pig but became downregulated at later stages with the exception of expression observed in the endocardium cell membrane and local blood vessels (Figs. 2 and 3). In the hiPSC cardiomyocytes, FLK1 was expressed on D1 and downregulated gradually thereafter, whereas PDGFR- α was expressed strongly for a longer period until D6 (Fig. 4A).

In the case of cardiac progenitor marker expression, we observed ISL1 expression across all investigated time points of the developing pig heart. It was expressed in the endocardium, myocardium and epicardium, as well

as the first heart field (FHF: the first wave of mesodermal cells that differentiate to form the initial heart tube and that express muscle-specific proteins) and the second heart field (SHF) derivatives (Figs. 2 and 3). Of note, during most stages, it was localized in the nucleus and cytoplasm, while at E70, ISL1 was only found in the cytoplasm (Suppl. Fig. 2). Furthermore, ISL1 expression was not limited to the embryonic heart but also expressed in other tissues, though not across the entire embryo, from E16 – E24 (Suppl. Fig. 3).

In the differentiating hiPSC, ISL1 was highly expressed across several time points from D1 to D6, but located in the cytoplasm at D1-D2 and changed to nuclear localization at D3-D6 (Fig. 4A).

Expression of NKX2.5 in the developing pig heart was present from E16 to E70. Importantly, we detected NKX2.5 in both the FHF and SHF derivatives, including the AT, AVC, VE and OFT. Moreover, we observed expression throughout the endocardium, myocardium and epicardium (Figs. 2 and 3). There appeared to be higher intensity on E18 and E22, however, this might be a technical artefact therefore we do not suggest these peaks bear biological meaning (Figs. 2 and 3). In the human cardiomyocyte model, heterogenous expression of NKX2.5 was observed in the D0 EBs and the expression increased thereafter. Interestingly, on D0 – D2 it was mostly detected in the cells within the outer layer of the EB (Fig. 4A).

In order to ensure that our antibody stainings revealed true expression of the proteins, we performed extensive antibody control stainings of the pig embryos and the EBs (as described in the Materials and Methods section). No specific staining patterns were observed in the pig embryos and no signal was detected in the EBs (Suppl. Fig. 4).

3.3. Comparative expression pattern of the immature cardiomyocyte markers CTNT, MYHC-B and mature cardiomyocyte marker CX43 in the developing pig heart and human cardiomyocyte model

In the pig heart, CTNT was detectable as early as E16 specifically in the ventral looping tube of the pig heart (Fig. 5). Expression was highest at E18, where it was found throughout the looping tube, as well as on E22, leveling off thereafter (Figs. 3 and 5). Interestingly, in the human cardiomyocyte model, CTNT was detectable at weak levels at early stages of development (D0 and D1) and became strongly expressed from D6 onwards (Fig. 4B).

The mature cardiomyocyte marker CX43 was highly expressed at several phases of development in the pig heart, including at E15, E20, E22, E24 and E70, whilst it was expressed at lower levels at E16 - E18 (Figs. 3,

5 and Suppl. Fig. 2). Expression was localized to the myocardium of the AT, VE, BC and OFT (Fig. 5). In our human cardiomyocyte model, we found expression at all investigated stages. However, on D0 and D7 it appeared to be localized to gap junctions and from D1 – D6 localized in the cytoplasm (Fig. 4B).

In the pig heart, MYHC-B was expressed highly from E16 to E22 and decreased to low levels at E24 and E70 (Figs. 3, 5 and Suppl. Fig. 2). At E16, MYHC-B was located within the looping heart, and at the later stages E18 - E24 in the myocardium of the AT, VE, BC and OFT (Fig. 5). At E70, MYHC-B was decreased within the AT and limited to the mature ventricle (Suppl. Fig. 2). Interestingly, we observed a differential pattern of expression in the human cardiomyocyte model, namely expression of MYHC-B only at D7 (Fig. 4B).

4. Discussion

In this study, we characterized various stages of pig embryonic heart development using morphological features and molecular markers. We asked whether the markers used to identify different stages of human cardiomyocyte differentiation should be revised and determined if molecular differences exist between mouse and pig cardiogenesis.

4.1 Anatomical features of early development of the pig heart

We report for the first time sequential anatomical events in early pig cardiac development, which recapitulate early cardiac formation events in other mammalian species. At E15/E16 the pig heart is a linear tube and starts looping at E18. At E20 the endocardial cushions and conotruncal cushions appeared and were followed by valve formation at E22. The outflow tract division became visible later at E24. Red blood cells were present from E18 onwards within the heart chambers suggesting the pig heart begins to pump later than E15, as previously reported [22]. Knowledge on this temporal timing of events will be useful for future studies investigating the early developing pig heart.

4.2 FLK1 is exclusively expressed in cardiac mesoderm but PDGFR- α is also expressed later on in cardiac progenitors

We found FLK1 to be a good mesodermal marker in the pig and that it was expressed early on in the differentiating hiPSCs as well. The expression of FLK1 in the pig is comparable to Flk1 expression in the

mouse, which is observed in the broad mesoderm around the primitive streak at E7.5 [30] and declines towards the end of gestation [31]. Therefore, FLK1 appears to be conserved in expression between humans, pigs and mice. Contrary, PDGFR- α was expressed also at later stages in the differentiating human hiPSC, co-expressing with cardiac progenitor markers (ISL1 and NKX2.5) and therefore should only be used as a cardiac mesoderm marker when used together with FLK1.

4.3 ISL1 is an excellent marker of cardiac progenitors but NKX2.5 is expressed throughout all stages

In the pig, ISL1 was expressed from E15 through to E24. Although ISL1 is a transcription factor and therefore expected to be detected only in the nucleus, we also found clear signals in the cytoplasm in the pig tissue as well as in the human EBs. Cytoplasmic expression of Isl1 has been previously shown in the mouse [33,34]. We speculate that cytoplasmic localization may regulate ISL1 function. In support of this, ISL1 was also detected in the cytoplasm at E70, a late stage where no ISL1 expression is expected. Lastly, we can also confirm that ISL1 is present in both heart fields as recently reported [35], disproving older studies of Isl1 as a second heart field marker [32]. In the differentiating hiPSC, ISL1 peaked on D2 in the nucleus, indicating the presence of cardiac progenitor cells at this stage of differentiation. With this, we can confirm with our study that ISL1 is a good marker of cardiac progenitors in large mammals.

Our finding that ISL1 was not only expressed in the heart but also in other tissues of the developing embryo overlaps with findings in the mouse. Here, Isl1 has been reported to be expressed during embryonic development in the pharyngeal mesoderm dorsal to the heart tube, the central nervous system, varying areas of the gastrointestinal tract, the hindlimbs, the umbilical cord and mesenchyme, the adrenal gland, thymus and facial tissues. It has also been detected in a wide range of tissues in the adult in several studies [36,37,46,38–45].

In the case of NKX2.5 expression in the pig, we observed expression from E16 – E70 throughout the heart, which is in accordance with Nkx2.5 expression in mice throughout gastrulation and adulthood [47] and confirms more studies in mice that revoke an older notion of NKX2.5 as an FHF marker [35]. Therefore, we confirm that NKX2.5 marks the progeny of both the FHF and SHF. In the hiPSC cardiomyocyte model we found that NKX2.5 was already expressed in a few cells from D0. This is in contrast to previous reports from mice, where NKX2.5 is expressed only in early heart progenitors [48] and at the cardiac progenitor stage in *in vitro* differentiation protocols after induction of ISL1 [49,50]. We previously reported low levels of NKX2.5 gene expression at D0

in the human EBs (four orders of magnitude lower than at D7) [26]. The expression we do observe in the *in vitro* human iPSC model might reflect spontaneous induction of the cardiac lineage at a very early stage. Single cell profiling of the EBs in the future might help confirm the genotype of these cells. Moreover, our immunostaining results showed that on D0, D1 and D2, NKX2.5 was predominantly expressed in the outer layer of the EBs, suggesting the presence of a differentiation wave, starting from the outside of the EB. Taken together, NKX2.5 was expressed at almost all stages in the pig heart and human cardiomyocyte model, supporting the notion that NKX2.5 is a good marker for the cardiac lineage that is conserved from mice to humans.

4.4 CTNT is a good marker for immature cardiomyocytes and MYHC-B marks both cardiac progenitors and immature cardiomyocytes

We detected CTNT in the pig in a similar time window as reported in mice, namely in the linear heart tube at E16 which corresponds to E8 in mice [51]. In the human cardiomyocyte model, however, it was detectable very weakly at D0 and D1 before it became strongly expressed at D6 and D7. Interestingly, previous studies on *in vitro* cardiac differentiation only report CTNT expression at later stages when the cells begin to show cardiomyocyte identity [2,52]. The weak expression of CTNT on D0 and D1 might indicate the presence of pre-cardiac mesoderm, which has been shown to express cTnT in the mouse on day E7.5 prior to heart tube formation [51]. In support of this, we have previously found that TNNT2 (the gene encoding CTNT) was expressed in human iPSC at weak levels on D0, D1 and D2 of our differentiation, however, increasing by four orders of magnitude at D7 [26]. When comparing the timely pattern of CTNT with other markers we found an intriguing difference between the pig and the human model: While in the pig, CTNT and NKX2.5 were upregulated together on E16 – 24, in the cardiomyocyte model CTNT was upregulated much later than NKX2.5. Many other *in vitro* studies have reported the same relationship [53–56], while others have observed a parallel regulation of CTNT and NKX2.5 as we detected in the pig [2,57]. This suggests that the human differentiation protocols themselves affect the expression pattern of the developmental markers. Our findings also suggest that the developmental pathways that take place in the developing heart are not replicated identically to *in vitro* differentiation methods.

We found the immature cardiomyocyte marker MYHC-B to be expressed from E16 – E24 in the looping heart and the myocardium of the pig and to be restricted to the ventricle at E70. Restriction to the ventricle has also

been reported in human fetal hearts [58]. Of note, in rodents MYHC-B expression decreases postnatally and is replaced by the variant MYHC-A [59]. Therefore, in contrast to humans, MYHC-A is the predominant form in the adult ventricle in rodents [14]. In this study, we only analyzed MYHC-B as we expected the expression in the pig to be more similar to humans than to rodents. Our data confirm this and show that MYHC-B is indeed restricted to the ventricle at later stages of pig development. Furthermore, we conclude that MYHC-B also marks cardiac progenitors and also specifically immature ventricular cardiomyocytes in the developing pig heart. In differentiating human PSCs, we and others detected MYHC-B only at the later stages of differentiation [2,3,60]. This disagreement of MYHC-B levels in cardiac progenitors between *in vitro* and *in vivo* cardiac cells has been shown before. MYHC-B was upregulated in fetal heart tissue compared to *in vitro* differentiated cardiomyocytes at three weeks after first contraction [12] and in a recent RNA-Seq study where human fetal heart progenitors from week 4 - 6 had 60-fold higher levels of MYHC-B expression than *in vitro* produced D3 progenitors [11]. This suggests that human PSC differentiation into cardiomyocytes may not faithfully replicate the expression of MYHC-B in the human fetus. To sum, MYHC-B expression seems to consistently differ between *in vivo* heart tissues and *in vitro* differentiation protocols and could therefore be used as a marker to improve *in vitro* protocols.

4.5 CX43 should be used with caution as a mature cardiomyocyte marker as it is also expressed in the mesoderm and immature cardiomyocytes in the developing heart

The mature cardiomyocyte marker, CX43, was evident in the mesoderm and immature and mature cardiomyocytes in the pig, while it was expressed mostly at early and not later time points in the human differentiating iPSCs. Interestingly, it was downregulated at the pig cardiac progenitor stages but well detectable in the differentiating hiPSCs at D3. Both the pig heart and human iPSC model expressed CX43 at the cardiac mesoderm stage. In contrast, CX43 is first expressed in the mouse heart at E9.5 while being detectable in other tissues much earlier [61–63]. Therefore, our data suggest that CX43 expression differs in the mouse compared to pigs and potentially humans, as suggested by our hiPSC data. However, this would have to be elucidated in detail in future studies.

At later stages of human fetal development, CX43 has been shown to be upregulated at the time when fetal cardiomyocytes align in the smooth helical organization needed for coordinated contraction of the ventricle and normal heart function [64]. Hence, it is used *in vitro* differentiation as a marker for mature and synchronized

cardiomyocytes [2–4,65,66]. In our hiPSC differentiation model, no maturation or synchronization step was included and we therefore expected low or absent expression of CX43 in the cardiomyocytes on D7. We suggest that CX43 should only be assessed when comparing expression levels between matured and immature cardiomyocytes, in order to determine the degree of maturation and synchronization. Importantly, the cells should be co-labeled with other stage-specific markers, such as sarcomeric proteins to confirm cardiomyocyte identity.

5. Conclusion

We have for the first time performed a temporal evaluation of commonly used markers for human cardiomyocyte differentiation protocols using a large mammalian model, the pig. We found that expression within the developing pig heart overlapped well with murine and human expression for the mesoderm markers FLK1 and the progenitor marker ISL1. Expression in the pig also faithfully recapitulated expression of the immature cardiomyocyte marker, MYHC-B and the mature cardiomyocyte marker CX43, when compared to the human. However, MYHC-B was also detectable in cardiac progenitor cells, immature cardiomyocytes and ventricular mature cardiomyocytes in both the pig and hiPSC model. Further, expression in the pig heart and the hiPSC model indicated that CX43 is also expressed in mesoderm and immature cardiomyocytes and not only in matured cardiomyocytes. Based on this study, we propose that a combination of FLK1+/PDGFR- α + would be valuable for identifying cardiac mesoderm, ISL1+/NKX2.5+ is a good combination for identifying cardiac progenitors and CTNT+/MYHC-B+ for marking immature cardiomyocytes. CTNT+/ISL1+ could be used to mark immature cardiomyocytes and CTNT+/ISL1- used to mark mature cardiomyocytes. CX43 remains an excellent marker for maturation and synchronization of cardiomyocytes, but should be used in combination of other sarcomeric proteins to confirm cardiomyocyte identity. This comparison of the expression of cardiogenesis markers in various species will lead to improved characterization of differentiating human cardiomyocytes in the future.

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403

404 Author Disclosure Statement

405 All authors declare that they have no competing interests.

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Figure 1: Pig heart developmental stages. **A)** Sagittal sections of HE-stained pig embryos at indicated days of embryonic development (E15 – E24) (upper panel) and magnifications of the heart area with depicted heart structures (lower panel). VE: Ventricle, OFT: Outflow tract, AT: Atrium, BC: Bulbus cordis, SV: Sinus venosus. *: conu-truncal cushions, little black arrow: endocardial cushions. Orientation arrows: A: Anterior, P: Posterior, D: Dorsal, V: Ventral. **B)** Table indicating the stages in embryonic days and key morphological features compared in the mouse, pig and human. ECC: Endocardial cushions.

Figure 2: Expression of early cardiac markers in the pig heart. A) Immunohistochemical images of FLK1, ISL1 and NKX2.5 in the pig heart at embryonic days E15 – 24 with overview images of the heart (upper row) and magnifications to see cellular distribution (lower row). Representative images of sections from three embryos per marker. VE: Ventricle, OFT: Outflow tract, AT: Atrium, BC: Bulbus cordis, SV: Sinus venosus. Scale bar for low magnification = 200 μ M, higher magnification images = 30 μ M.

Figure 3: Marker expression levels during pig embryonic heart development. Reciprocal intensity values from immunohistochemical images displaying strong, normal, weak or no expression. Predominant location of each marker is indicated (Heart tissue as well as cellular location). Average values of all slices from three pig embryos.

Figure 4: Expression of cardiac markers in the human cardiomyocyte model. A)

Immunohistochemical images of early cardiac markers FLK1, ISL1, NKX2.5 and PDGFR- α in differentiated human iPSCs from day D0 – D7 of differentiation. B) Immunohistochemical images of late cardiac markers

CTNT, CX43 and MYHC-B in differentiated human iPSCs from day D0 – D7 of differentiation. Counterstaining with Hoechst (blue) for DNA. Representative images of 2 - 3 embryoid bodies per marker.

Scale bar = 500 μ m.

Figure 5: Expression of late cardiac markers in the pig heart. A) Immunohistochemical images of CTNT, CX43 and MYHC-B in the pig heart at embryonic days E15 – 24 with overview images of the heart (upper row) and magnifications to see cellular distribution (lower row). Representative images of sections from three embryos per marker. VE: Ventricle, OFT: Outflow tract, AT: Atrium, BC: Bulbus cordis, SV: Sinus venosus. Scale bar for low magnification = 200 μ M, higher magnification images = 30 μ M.

Supplementary material

Table 1: Cardiomyocyte differentiation media

mTeSR-ROCK	
mTeSR1 (STEMCELL Technologies 85850)	10 ml
ROCK (5 mM, Cayman Chemical 10005583)	20 µl
PSG (Life technologies 10378016)	100 µl
DO medium	
KO-DMEM	10 ml
PSG (Life technologies 10378016)	100 µl
ITS (BD 354351)	10 µl
Y (10 mM, Abcam 120129)	10 µl
FGF2 (10 µg/ml, Peprotech 100-18B)	10 µl
Activin A (10 µg/ml, eBioscience 34-8993-85)	10 µl
CHIR (1 mM, Axon Medchem 1386)	25 µl
BMP4 (10 µg/ml, R&D 314-BP-010)	1 µl
TS medium	
KO-DMEM (Life technologies 10829018)	10 ml
PSG (Life technologies 10378016)	100 µl
TS *	100 µl
Asc (250 mM, Sigma-Aldrich 49752)	10 µl
Wnt medium	
KO-DMEM (Life technologies 10829018)	10 ml
PSG (Life technologies 10378016)	100 µl
TS *	100 µl
Asc (250 mM, Sigma-Aldrich 49752)	10 µl
Wnt (2mM, Tocris Cat.-No. 5148)	20 µl

*TS is prepared by adding 1ml of sodium selenite (27 mg sodium selenite Sigma-Aldrich S5261 in 400 ml PBS w/o Ca and Mg) to the 99 ml transferrin (55 mg transferrin Sigma-Aldrich T8158 in 99 ml PBS w/o Ca and Mg)

Table 2. Reciprocal intensity values with their corresponding integer number used to create the heat map and the heat map label.

Reciprocal intensity values	Integer number	Heat map label
0 - 50	0	No expression
50 - 120	2	Weak expression
120 - 170	4	Normal expression
170 - 250	6	Strong expression

Supplementary figure 1: Pluripotency markers in D0 EBs. Immunohistochemical images of pluripotency markers SSEA3, OCT4 and SOX2 in EBs on D0 of the differentiation protocol.

Supplementary figure 2: Marker expression in the E70 pig embryo heart. Immunohistochemical images of FLK1, ISL1, NKX2.5, CTNT, CX43 and MYHC-B in the pig heart at embryonic day E70 with overview images of the heart (upper row) and magnifications to see cellular distribution (lower row). Scale bar for low magnification = 200 μ M, higher magnification images = 30 μ M.

Supplementary figure 3: Overview images of the entire embryo at stages E16 – E24.
Immunohistochemical images for Isl1.

A

B

Supplementary figure 4: Negative controls of antibody stainings. A) For immunohistochemistry stainings of pig embryos at indicated stages. The following antibody controls were used: Negative mouse IgG1, negative mouse IgG2b and normal rabbit IgG. **B)** For immunocytochemistry stainings of D0 EBs, only secondary antibodies were used.