CHAPTER SEVEN

Multipotent NKX2-5⁺ cardiac progenitors derived from human embryonic stem cells

Stefan R. Braam¹, David A. Elliott²,⁴, Elizabeth S. Ng², Dorien Ward van Oostwaard¹, Katerina Koutsis², Tanya Hatzistavrou², Louise Lagerqvist³, John M. Haynes³, Colin W. Pouton³, Leon Tertoolen¹, Robert Passier¹, Christine L. Mummery¹, Andrew G. Elefanty², Edouard G. Stanley²

¹ Monash Immunology and Stem Cell Laboratories, Monash University, Victoria, Australia
² Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands
³ Monash Institute of Pharmaceutical Science, Monash University, Victoria, Australia
⁴ These authors contributed equally

Manuscript in preparation
Cardiac progenitor cells give rise to the major cellular components of the heart. Understanding how they form and what they can become has important implications for elucidating the mechanisms underlying (abnormal) heart development. In addition, the ability to isolate these cells from pluripotent stem cells allows characterization of their properties and possibly expansion in culture, a crucial step for advancing cardiac translational medicine. Functional analysis of these cells has been hampered by the paucity of lineage specific markers and inefficient, undefined differentiation procedures. Here we describe the targeted modification of two independent human embryonic stem cell (hESC) lines in which EGFP was inserted into the locus of Nkx2–5, one of the earliest transcription factors expressed in heart development. EGFP fluorescence driven by the endogenous Nkx2–5 promoter faithfully reported cardiovascular lineage commitment of differentiating hESC under defined culture conditions. The early Nkx2–5 positive cell population contained multipotent progenitor cells capable of directed differentiation to cardiomyocytes, endothelial and vascular smooth muscle cells. Nkx2.5 overlapped only partially with previously reported selection markers for the cardiovascular lineage. These data are compatible with expectations from heart development and identify a progenitor that can give rise to the three major cell types of the heart or through fine temporal fine tuning, almost exclusively cardiomyocytes. Taken together, these experiments demonstrate the utility of hESC for analyzing the previously inaccessible events of human cardiac lineage specification.
Introduction

The ability to generate functional cardiovascular (progenitor) cells from human pluripotent stem cells is beginning to offer unprecedented opportunities for translational research in cardiac medicine. Applications range from the use of cells to study early human cardiac development, the development of disease models, predictive cardiotoxicity assays, drug development and ultimately the development of cell therapy for degenerative diseases. Advance of all of these applications depends on robust differentiation procedures underpinned by detailed knowledge of the signaling mechanisms regulating cell fate.

Over the previous decade methods for expansion and directed differentiation of human embryonic stem cells (hESC) has improved considerably. One recurring theme is that most successful differentiation protocols recapitulate the regulatory circuits that control normal development. Initially, protocols supporting cardiomyocyte differentiation relied on poorly defined reagents and inefficient cell culture methods. This resulted in cultures with only a very small percentage of beating cells (<1%). Later it was shown that co-culture with endoderm-like cells, mimicking signaling from endoderm in normal development resulted in a relatively efficient and robust cardiomyocyte differentiation. More recently, several groups have shown that the cytokines Bone Morphogenetic Protein 4 (BMP4) and Activin A induce mesendoderm in differentiating hESC cultures. However, the exact timing, concentration and spectrum of growth factor requirements for the initiation of cardiogenesis, formation of cardiac progenitors and further maturation remain to be defined.

In mouse the earliest cardiac progenitor identified, arises in the primitive streak and expresses Flk-1/KDR. Later in development at least two progenitor populations are present. The first population expresses the transcription factor Nkx2-5 and is present in the cardiac crescent or the first heart field (FHF). Nkx2-5 is one of the earliest cardiac progenitor markers and continues to be expressed in the adult myocardium. The committed FHF progenitors contribute to left and right ventricles, both atria and AV canal. The second population, known as the second heart field (SHF) is characterized by the expression of the transcription factor Isl1 in addition to Nkx2-5. These cells give rise mainly to the outflow tract and right ventricle but also contribute to all other regions of the heart except the left ventricle. In the developing SHF, multipotent Isl1+/Nkx2-5+ progenitors can differentiate to endothelial cells, vascular smooth muscle and cardiomyocytes.

Two studies to date have used a human pluripotent stem cell model for identification, selection and characterization of cardiac progenitors. An antibody mediated cell sorting method developed in mice and based on cell surface expression of the VEGF-receptor FLK-1/KDR was instrumental in the first isolation human cardiovascular progenitors from hESC. Later, genetic tagging of Isl1 allowed the isolation of another population of human cardiac progenitor cells from hESC. Although both methods worked effectively neither was an exclusive cardiac progenitor marker. Flk-1/KDR is not specific for cardiac mesoderm and ISL1 is broadly
expressed in multiple non-cardiac lineages during early development.

Here we report targeted modification of NKX2-5 with EGFP in hESC. The activity of the reporter faithfully recapitulated the process of cardiac development and allowed purification of cardiomyocytes from a heterogeneous cell population. The transgenic line permitted development of a cytokine screening strategy in defined medium that ultimately resulted in an optimized cardiac differentiation protocol. FACS based purification of early NKX2-5 positive cells revealed a multipotent progenitor population capable of directed differentiation to the cardiac, endothelial and vascular smooth muscle cell lineages.

Materials and methods

hESC culture and differentiation
hESC culture and gene targeting were performed as previously described. Differentiation was induced by forced aggregation in APEL media, with the following cytokines: 20 ng/ml of BMP4 (R&D) and Activin A (R&D), 30 ng/ml VEGF (R&D) and 40 ng/ml SCF (R&D). hESC-CMs were also derived using the END2 co-culture system, as previously described.

Flow cytometry
Embryoid bodies (EBs) were dissociated using TrypLE Selectä (Invitrogen), and intracellular flow cytometry carried out on cells fixed and stained with anti-ISL1 (DSHB, clone 39.4D5), anti-OCT4 (Santa Cruz Biotechnology) and anti-NKX2.5 (Abcam) antibodies as previously described. Mouse anti-human primary antibodies reacting with cell surface antigens for live cell sorting included unconjugated anti-E-CADHERIN (Zymed), anti-TRA-1-60 (Chemicon), anti-SSEA4 (Chemicon), FITC-conjugated anti-CD9 (BD Biosciences), anti-PDGFα (BD Biosciences), anti-VCAM (BD Biosciences) and phycocerythrin (PE) conjugated anti-CXCR4. Non-conjugated antibodies were detected with allophycocyanin (APC)-conjugated goat anti-mouse IgG (BD Biosciences). Flow cytometric gates were set using control cells labeled with the appropriate isotype control antibody. Single cell cloning was performed using the single cell deposition function of a FACSaria FACS station as previously described.

Imaging
Cells were cultured on glass coverslips, fixed in 4% PFA for 30 min, permeabilized for 5 min. with 0.1% Triton X-100 in PBS and blocked with 4% normal goat serum. Cells were incubated with primary antibodies and labeled with alexa405, Cy3, Cy5 and/or alexa647 conjugated secondary antibodies. Primary antibodies used in this study were against α-actinin (clone EA53, Sigma), PECAM (DAKO), smooth muscle actin (Abcam), NKX2-5 (Santa Cruz) MLC2V (Synaptic Systems), MLC2A (Synaptic Systems), MYH6 (Chemicon), GATA4 (Santa Cruz), c-KIT(Dako), and PDGRα (BD) Nuclei were counter stained with DAPI. Confocal scanning was performed on a Leica SP5 confocal laser scanning microscope (40x and 63x oil immersion objectives). For live cell imaging, cells were cultured on MatTek dishes (MatTek Corp. Amsterdam) and imaged...
on a Leica SP5 using the resonance scanner at a resolution of 512x256 (30fps). For calcium imaging, cells were loaded with Fura Red (Molecular Probes, Invitrogen). The 488nm laser was used to excite EGFP and Fura Red (calcium free). Two PMTs were used simultaneously for detecting EGFP and Fura Red specific photons respectively. ImageJ imaging processing software was used for normalization of Fura Red fluorescence to EGFP and for further data analyses.

**ELECTROPHYSIOLOGY**

Patch clamp electrophysiology was carried out essentially as described previously, with minor modifications\(^8\). Briefly, beating clumps of cardiomyocytes were dissociated using Tryp.Le, replated on gelatin-coated coverslips and measured in current clamp mode between 7 and 14 days after plating.

For MEA electrophysiology, hESC-CM clusters were microdissected and replated on plasma cleaned fibronectin coated 60 electrode MEAs. Extracellular recording was performed using a MEA1060INV MEA amplifier (Multi Channel Systems, Reutlingen, Germany) at 37°C. Output signals were digitized at 10 kHz by use of a PC equipped with a MC-card data acquisition board (Multi Channel Systems, Reutlingen, Germany).

**FORCE MEASUREMENTS**

A beating region within an embryoid body was selected and viewed through a Nikon Eclipse TS100 inverted microscope with Hoffman modulation contrast optics, coupled to a Basler (model A602f) camera. Quick Caliper (SDR Clinical Technologies) enabled the acquisition of a 10 second capture (80 frames/second). Prior to the addition of agonists/activators a 10 second capture was obtained to establish baseline conditions. Following this, either forskolin (1μM), endothelin-1 (ET-1; 10nM) or vehicle controls were added to the well, and the plate placed back in a humidified incubator (37°C, 5% CO\(_2\)). A second 10 second capture was then taken 2 and 5 minutes post forskolin or ET-1.

ET-1 was dissolved in physiological salt solution (PSS) buffer consisting of (in mM) NaCl 140, KCl 6, CaCl\(_2\) 2, MgCl\(_2\) 1, HEPES 20 and glucose 10, supplemented with 1.4 % (w/v) BSA (pH 7.4, 37°C). Forskolin was dissolved in ethanol and the final ethanol concentration did not exceed 0.01% of the media volume in the well.

The video captures were analysed using the Metamorph® Imaging System (Molecular Devices Ltd). For each image stack constituting a 10 second capture, an intensity threshold was set for the beating image. The Metamorph® software was then used to track the intensity centroid of a selected particle throughout the 10 second image stack. The amplitude of contraction of the selected particle (in pixels) was plotted against time for each time point (See Figure 7.3C). The number of peaks within the 10 second capture was converted to beats per minute to represent the contraction frequency. The frequency recorded in response to an agonist was reported as a percentage of the basal spontaneous rate (i.e. prior to agonist addition).
As previously reported hESC-derived cardiomyocytes, not all beating aggregates responded to agonist stimulation. Responding and non-responding aggregates were identified using a repeated measures ANOVA test of the time interval between beats in each 10 second capture. Differences between means were tested using the Student’s t-test and values of p<0.05 were considered statistically significant.

Results

**NKK2-5EGFP/w HESC LINE FACILITATES IDENTIFICATION AND PURIFICATION OF HUMAN CARDIAC PROGENITOR CELLS**

To investigate early differentiation events in human cardiogenesis we introduced sequences encoding the green fluorescent protein (EGFP) into the NKX2-5 locus of hESC (Figure 7.1). The targeting vector contained a PGK promoter driving expression of neomycin acetyltransferase flanked by loxP sequences, the recognition sites of bacteriophage Cre recombinase. Correctly targeted hESC clones were identified by polymerase chain reaction (PCR) amplification (Figures 7.1A & data not shown) and sequencing of the resultant PCR products. The selection cassette was removed by transient transfection of hESC with a Cre recombinase expression vector and subsequent single cell deposition by FACS7. Cassette deletion was validated by PCR (data not shown). Southern blotting with an EGFP probe confirmed that the identified NKK2-5EGFP/w clones contained only a single integration event (Figure 7.1B). Two hESC lines, HES319 and MEL1 (Millipore) were targeted in this manner to generate H3 NKK2-5EGFP/w and M1 NKK2-5EGFP/w, respectively. All NKK2-5EGFP/w clones expressed markers of undifferentiated HESC, were karyotypically normal and formed teratomas (data not shown). To derive cardiomyocytes NKK2-5EGFP/w cells were initially differentiated20 in the END-2 co-culture system. As expected, this protocol resulted in reproducible and robust cardiac differentiation. Importantly, eGFP driven by the NKK2-5 promoter was specifically activated in beating foci. Beating areas could be readily identified based on bright green fluorescence and all green beating foci stained positive for cardiac α-actinin (Figure 7.1C). To verify that EGFP expression recapitulates endogenous NKK2-5 expression, dissociated EGFP+ cells were stained with anti-NKX2-5 antibody. All EGFP+ cells showed prominent nuclear NKX2-5 staining, as well as expression of the cardiac protein a-actinin (Figure 7.1D). Furthermore, dissociated cardiomyocytes stained positive for the cardiac structural proteins MLC2a, MLC2v and beta myosin heavy chain as well as transcription factors NKX2-5, ISL1 and GATA4 (data not shown). EGFP cardiomyocytes displayed a similar fetal sarcomeric organization as cardiomyocytes from control hESC lines21.

**OPTIMIZED DIFFERENTIATION IN DEFINED MEDIA**

Having validated the integrity of the GFP reporter cell lines and retention of the ability to form cardiomyocytes robustly, we designed a cytokine screening strategy based on the fluorescent reporter as a read-out. The aim was to develop a method for directed cardiovascular differentiation of hESC under chemically defined culture conditions. To screen and cross titrate these factors properly without interference from undefined components in co-cultures we
**Figure 7.1**

Generation and characterization of NKX2-5EGFP/w knock-in lines

(A) Design of the gene targeting vector to insert sequences encoding EGFP into exon 1 of the NKX2-5 locus. NeoR is the PGK-Neo cassette encoding neomycin acetyltransferase, flanked by LoxP sites (black triangles) (B) Southern blot with a GFP probe to confirm a single integration in the genome. (C) α-actinin staining on cardiac differentiated H3 NKX2-5EGFP/w (D) co-staining for NKX2-5 and α-actinin on dissociated H3 NKX2-5EGFP/w.

**Figure 7.2**

Cardiac differentiation in defined APEL medium

(A) Optimized differentiation protocol based on temporal addition of BMP, SCF, Activin A and SCF for three days. (B) Temporal expression pattern of NKX2-5 during EB differentiation by flow cytometry for EGFP+ cells (C) Immunofluorescence of NKX2-5 reporter gene expression.
used a forced aggregation (or “spin EB”) protocol in a chemically defined media (APEL)\textsuperscript{16,17} supplemented with BMP 4 and Activin A\textsuperscript{5,6}. Since cardiac mesoderm derives from a cell population expressing the VEGF receptor, KDR\textsuperscript{6}, and stem cell factor (SCF) has been shown to improve cell viability in spin EBs\textsuperscript{22} both VEGF and SCF were included in the differentiation medium (Figure 7.2A).

Optimal cytokine concentrations were determined by cross titration of the growth factors mentioned above, examination of EBs for eGFP expression and scoring for the appearance of spontaneous contractile foci. The optimized protocol presented here consists of two initial steps performed in suspension culture; i) mesendoderm induction by a combination of BMP4 and Activin A until day 3 of differentiation and ii) further specification of cardiac mesoderm relying on endogenous signaling networks established by the primitive streak–like population generated in step I (Figures 7.2A & S7.2). Removal of growth factors on day 3 leads to a significant increase in the production of NKX2–5EGFP\textsuperscript{2} cells (Figure 7.2). This suggests that by day 3 EBs contain sufficient patterning information for cardiomyogenesis to proceed autonomously without the addition of exogenous factors.

The temporal expression pattern of NKX2–5 during EB differentiation was established by flow cytometry for EGFP\textsuperscript{+} cells. Control experiments using FGF2 demonstrated a requirement for mesendoderm induction to allow cardiac differentiation to proceed (Figure 7.2B). EGFP\textsuperscript{+} cells emerged on days 6–7 of differentiation and increased as a percentage of the culture until day 12 (Figure 7.2B). After transfer to adherent culture beating foci appeared within EBs. NKX2–5EGFP\textsuperscript{+/w} EBs maintained for 90 days continue to contract; however, after day 12 little increase was observed in percentage of EGFP\textsuperscript{+} cells. To confirm that EGFP expression recapitulated NKX2–5 expression, sorted EGFP\textsuperscript{+} cells were stained with anti-NKX2–5 antibody. All EGFP\textsuperscript{+} cells examined showed significant nuclear NKX2–5 staining, as well as expression of cardiac $\alpha$–actinin (data not shown).

**NKX2–5EGFP\textsuperscript{+/w} cells can differentiate to functional cardiomyocytes**

For many future applications requiring human cardiomyocytes derived from stem or progenitor cells, it will be important to know their electrical and functional properties. We therefore analyzed the action potentials of cardiomyocytes generated from the H3 NKX2–5EGFP\textsuperscript{+/w} line. Cells were classified as atrial–like, ventricular–like or pacemaker–like based on the profiles of the action potentials, as previously. As in cardiomyocytes from the parent HES3 line induced by END2 cell co-culture, the majority of cardiomyocytes from the NKX2–5EGFP\textsuperscript{+/w} cell line displayed a ventricular–like action potential; only a minority of cells displayed characteristics of pacemaker– or atrial cells\textsuperscript{18}. The average upstroke velocity was 6,4±2,3 V/s , the 90% repolarization 522,8±199,6 ms, the amplitude 88,8±6,6 mV and the resting potential was $-43,5\pm7$ mV in 18 independent measurements, A representative ventricular like action potential is shown in Figure 7.3A. Next the cells were analyzed using Multielectrode array (MEA) field potential analyses. As expected the cells showed a trace typical for hESC-CM (Figure 7.3B)\textsuperscript{21}. In summary, the NKX2–5 heterozygous cells were indistinguishable from unmodified
Figure 7.3
Characterization of NKX2-5EGFP/w cardiomyocytes

(A) Patch clamp electrophysiology showing a representative ventricular like action potential from H3 NKX2-5EGFP/w, (B) Field potential from a cluster of contracting cardiomyocytes measured on a multi electrode array (recording from one electrode). (C) Fura red calcium imaging using confocal microscopy from a beating cluster. (D) Contraction force measurements of beating EBs in response to ET-1 and Forskolin.
hESC-CM with respect to their electrical phenotype. To investigate properties of the cells in more detail, they were labeled with Fura Red to visualize the calcium homeostasis. As expected the cells showed typical calcium traces that accompanied contraction of the cells, indicating normal calcium homeostasis (Figure 7.3C, movie S7.2). Finally the cells were tested functionally by adding the ionotrophic/chronotrophic agents endothelin-1 (ET-1) and Forskolin. Both compounds exert similar effects on cardiomyocytes but the mechanisms are different: ET-1 increases the concentration of cytosolic Ca^{2+} while Forskolin activates adenyl cyclase resulting in elevated cAMP levels. Approximately 57% (4/7) of the spontaneously beating cardiomyocyte aggregates responded to ET-1 (10nM) addition, whereas 66% (2/3) responded to forskolin (1μM) addition. In the responding aggregates, ET-1 (10nM) increased contraction frequency by 58 ± 11% (p<0.05, students t-test, n=4). Preliminary investigations with forskolin indicated that it, also, increases contraction frequency (by 43±7%; n=2). Although the responses were not homogeneous, the hESC-CM do contain functional endothelin receptors, as well as intact cAMP signalling pathways, both of which are involved in the modulation of spontaneous contraction frequency.

**ISOLATION AND CHARACTERIZATION OF MULTIPOTENT NKX2-5\(^{+}\) CARDIAC PROGENITOR CELLS (CPCs)**

Having demonstrated the cardiomyogenic potential, the validity of the reporter and the functionality of cardiomyocytes generated from H3 NKX2-5EGFP/w we used the line to investigate the temporal dynamics of the appearance of NKX2-5 positive cells in greater detail. Studies in mouse embryos and mESC have shown that early Nkx2-5 positive cells co-express Flk-1 and c-kit. These cells have the potential to differentiate into cardiomyocytes, vascular smooth muscle and endothelial cells\(^{23}\). To determine whether this is also the case for early NKX2-5\(^{+}\) cells from hESC we analyzed the NKX2-5\(^{+}\) cells at day 7 of END-2 co-culture for the membrane markers c-Kit, PDGFR\(\alpha\) and Flk-1/KDR by confocal imaging (Figure 7.4A). While PDGFR\(\alpha\) was expressed at low levels we could find no evidence for c-Kit expression or Flk-1/KDR (Fig 7.4A and data not shown). Many cells that expressed high levels of PDGFR\(\alpha\) did not express NKX2-5 (Figure 7.4A, see insert), thus did not correspond with a cardiac progenitor population. Furthermore, it was clear that none of these markers segregated exclusively with the NKX2-5 population. Analyses of day7 cells derived from spin EB indicated the presence of a comparable population. PDGFR\(\alpha\) was expressed at low levels and Flk-KDR expression did not exceeded background levels. We next sorted day 7 NKX2-5\(^{+}\) cells from H3 NKX2-5EGFP/w differentiated in End-2 co-culture using flow cytometry (Figure 7.4C). Cells were re-plated on fibronectin coated substrates in defined culture medium supplemented with selected recombinant growth factors. To date, most CPC differentiation protocols have not used defined media; as a result, the signaling pathways required for cardiovascular lineage specification have not been precisely defined. Addition of bFGF plus BMP4 resulted in the appearance of SMA and PECAM positive cells as well as \(\alpha\)-actinin positive cells (Figure 7.4D), suggesting that the sorted NKX2-5\(^{+}\) cell population contained multi-lineage cardiovascular progenitors. The NKX2-5\(^{-}\) cells did not form these cells under the same conditions indicating the NKX2-5 defined the cell population with cardiovascular differentiation potential more precisely than
Isolation and directed differentiation of NKX2-5 progenitor cells

(A) Confocal microscopic imaging of END-2 co-cultures at day 7 of differentiation. NKX2-5\(^+\) areas stained for PDGFR\(\alpha\) and c-Kit. (B) FACS analyses of day 7 spin-
EB differentiations stained for PDGFR\(\alpha\) and Flk-1/KDR (gated on NKX2-5\(^+\) cells) (C) experimental flowchart describing FACS sorting
NKX2-5 progenitors (D) directed
differentiation of END-2 derived
CPCs to endothelial lineages (positive
for PECAM), vascular smooth muscle
(\(\alpha\)-smooth muscle actin) and
cardiomyocytes (\(\alpha\)-actinin)

Figure 7.4

Isolation and directed differentiation of NKX2-5 progenitor cells
the other markers tested. Interestingly, the balance between FGF and BMP signaling has been described as a fate regulator of pericardial chick mesoderm, determining the choice between the epicardial and myocardial lineages\textsuperscript{24,25}. We therefore cross-titrated these two growth factors in the sorted NKX2.5-GFP cells and determined their cardiac differentiation potential. Preliminary results indicated that BMP alone gives rise to cultures highly enriched for contracting cardiac α-actinin positive cells. Addition of bFGF led to a clear dose dependent lineage shift to non-myocardial cells, suggesting that the balance between BMP and FGF regulates the fate of the NKX2-5\(^+\) CPC population from hESC as in the chick.

Discussion

The origin and fates of human cardiovascular progenitor cells are still largely unknown. Here we show that the combined use of pluripotent stem cells and gene targeting allows dissection of previously inaccessible events during human cardiac progenitor development. Insertion of eGFP in the NKX2-5 gene resulted in two independent hESC lines that captured cells with cardiovascular developmental potential from the heterogeneous mixtures of hESC derivatives. Beating foci were brightly fluorescent and eGFP positive areas stained positive for both cardiac α-actinin and NKX2-5 proteins. Electrophysiological analysis, calcium imaging and force measurements confirmed their phenotype as functional cardiomyocytes, indistinguishable from cardiomyocytes generated from wild type hESC. The bright eGFP reporter activity driven by the endogenous NKX2-5 promoter line permitted development of a cytokine screening strategy that ultimately resulted in an optimized cardiac differentiation protocol. This new protocol makes use of defined medium supplemented with the cytokines BMP, Activin A, SCF and VEGF. Notably, removal of the growth factors at day 3 led to a significant increase in the production of NKX2-5\(^{\text{EGFP}^-}\) cells (up to 35%), suggesting that by day 3 the EB contains sufficient patterning information for cardiomyogenesis to proceed without further addition of exogenous factors. Recent work on mESC suggests that EBs can be polarized in an anterior-posterior direction and form a structure that resembles the primitive streak. Local activation of the Wnt pathway resulted in an epithelial–to–mesenchymal transition and differentiation into mesendodermal structures. This process required exogenous factors but was self-reinforcing after initiation\textsuperscript{26}. A similar process seemed to occur in our protocol despite the absence of exogenous Wnt signals. Addition of fresh cytokines at later time-points never enhanced differentiation outcome. This suggested that the auto- and endocrine signaling between the different cell types is so balanced that alteration of exogenous signaling mainly disturbs their differentiation potential.

Isolation of human cardiac progenitors based on NKX2-5 expression could be a good approach to dissect cardiac progenitor lineage decisions without interference of cells not associated with this lineage. In the mouse, Nkx-2.5 transcripts are first detected at early headfold stages in myocardioogenic progenitor cells. Expression precedes the onset of myogenic differentiation, and continues during lifetime of a cardiomyocyte\textsuperscript{9}. NKX2-5 is one the earliest
genes that control cardiac differentiation and is present in both the progenitors of the FHF and SHF. Transcripts are mainly expressed in the heart although expression is also detected in the cardiac associated pharyngeal endoderm, lingual muscle, spleen and stomach. In the mouse several cardiac progenitor populations have been described and the majority of them expressed Nkx2-5. Only the Flk-1 progenitor described by Kattman et al emerges at such an early developmental stage that Nkx2-5 is not yet expressed. However according to fate mapping experiments performed in mouse embryos, Flk1+ progenitors contribute only to a subset of cardiac muscle cells. Moreover Flk-1 is not expressed in all cells of the cardiac crescent during development, nor is it cardiac specific. Two independent groups have described the isolation of putative primordial / FHF specific progenitors in the mouse. Both of these studies made use of a Nkx2-5 reporter construct for the isolation of CPCs. However there were differences between the cell populations. The cells identified by Wu and colleagues co-expressed c-Kit, were bi-potent, indicated by their differentiation to cardiomyocytes and smooth muscle cells. The cells identified by Christoforou and colleagues co-expressed c-kit and Flk-1/KDR and were reported to be multipotent: besides cardiomyocytes and vascular smooth muscle cells they were also capable of forming endothelial cells. Whether the cells from these two studies represent the same population remains to be investigated. In the developing SHF a similar multipotent cell can be identified. This progenitor expresses Isl1+/Nkx2-5+ and is capable of directed differentiation to endothelial, vascular smooth muscle and cardiomyocyte lineages.

Isolation of NNX2-5+ cells from the H3 NNX2-5EGFP line at day 7 of differentiation resulted in multi-lineage differentiation to cardiomyocytes, endothelial cells and vascular smooth muscle cells. These findings indicate a differentiation potential comparable to the mouse multipotent progenitors found in the SHF, and the Nkx2-5 progenitors described by Christoforou et al. However, in contrast to the mouse Nkx2-5 CPCs our cells do not express c-Kit, and FLK-1 is only expressed at low levels, which suggests a difference to the mouse cells described by Christoforou. Furthermore the relationship to ISL1 remains largely unclear. According to Bu et al (2009) the expression of ISL1 in human cardiac development precedes NNX2-5 expression, however this was not investigated in detail. Although some of our NNX2-5+ cells do express ISL1 it is certainly not co-expressed in all cells. In general, from studies in the mouse it seems that ISL1 and NNX2-5 mark a distinct but overlapping population of cardiac progenitors. However it is of note that ISL1 is expressed in multiple organs and tissues in the developing embryo, including motor neurons and pancreas progenitors. In accordance with this, only 4.2% of the human Isl1 clones gave rise to cells expressing cardiac genes, 3.1% to endothelial and 44.1% to smooth muscle.

Further follow up studies including clonal assays on human NNX2-5 positive cells, will be required to confirm the multipotency of our cells and their relationship with ISL1. Finally, it should be mentioned that analyses of the ISL1 family of cardiac progenitors suggests clear differences in mouse and human cardiac development. In the mouse, a rapid transition from an Isl1 progenitor to migration and conversion to cells of the SHF is observed.
Consequently, later in development relatively few Isl1-expressing cells can be found in the heart. Furthermore, Isl1-progenitors generally co-express the transcription factors Nkx2-5 and Flk-1/KDR; indicative of a rapid fate restriction. In the first trimester of cardiac human development relatively large numbers of ISL1 expressing cells are present that do not co-express KDR or Smooth muscle myosin heavy chain or cardiac Troponin, perhaps reflecting the fact that heart development in humans is a much longer process than rodents. It has been suggested that these cells represent the upstream precursor for the multipotent progenitors in the SHF lineage\textsuperscript{13}. Therefore the presence of a family of ISL1 expressing intermediate, lineage-restricted progenitors could reflect the requirement for the more extensive cell expansion necessary to produce cell numbers orders of magnitude greater in adult human hearts than in mice\textsuperscript{10}.

In summary, the findings reported here demonstrate the combined potential of the pluripotent stem cell model with genetic manipulation of these cells. Targeted modification of NKX2-5 with EGFP resulted in a highly specific reporter line that can be used for the isolation and lineage dissection of human cardiac progenitors. Specifically we show that day 7 CPCs are capable of directed differentiation to cardiomyocytes, vascular smooth muscle and endothelial cells. Interestingly it seems that the ratio of exogenous bFGF to BMP determines the differentiation fate of the NKX2.5 marked CPCs. BMP2 is a known cardiogenic factor expressed in lateral plate mesoderm and cardiac-associated pharyngeal endoderm\textsuperscript{31}. Our data suggests that this signaling might be a driver of CPC specification to cardiomyocytes. It is expected that further studies on CPCs will eventually lead to optimized culture conditions for self-renewal and directed differentiation to various cardiovascular lineages of the heart.

**ACKNOWLEDGEMENTS**

We thank J Monshouwer Kloots for technical support, the LUMC flow cytometry core for flow cytometric assistance and the Molecular Cell Biology LUMC Imaging core for access to their imaging equipment. This work was financially supported by the Bsik Dutch Platform for Tissue Engineering and EU Heart Repair FP6 (S.R.B.).
References


