CHAPTER 3

Dual reporter MESP1$^{mCherry/w}$-NKX2-5e$^{GFP/w}$ hESCs enable studying early human cardiac differentiation

Sabine C. Den Hartogh[1], Chantal Schreurs[1], Jantine J. Monshouwer-Kloots[1], Richard P. Davis[1], David A. Elliott[2], Christine Mummery[1], Robert Passier[1].

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1. Department of Anatomy and Embryology, Leiden University Medical Centre, Leiden Postal zone S-1-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands
2. Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, Victoria, Australia

ABSTRACT

Understanding early differentiation events leading to cardiogenesis is crucial for controlling fate of human pluripotent stem cells and developing protocols that yield sufficient cell numbers for use in regenerative medicine and drug screening. Here, we develop a new tool to visualize patterning of early cardiac mesoderm and cardiomyocyte development *in vitro* by generating a dual MESP1mCherry$^{/w}$-NKX2-5$^{eGFP/w}$ reporter line in human embryonic stem cells (hESCs) and using it to examine signals that lead to formation of cardiac progenitors and subsequent differentiation. MESP1 is a pivotal transcription factor for pre-cardiac mesoderm in the embryo, from which the majority of cardiovascular cells arise. Transcription factor NKX2-5 is expressed upon cardiac crescent formation. Induction of cardiac differentiation in this reporter line resulted in transient expression of MESP1-mCherry, followed by continuous expression of NKX2-5-eGFP. MESP1-mCherry cells showed increased expression of mesodermal and EMT (epithelial-mesenchymal-transition) markers confirming their mesodermal identity. Whole-genome microarray profiling and FACS analysis of MESP1-mCherry cells showed enrichment for mesodermal progenitor cell surface markers PDGFR-α, CD13, and ROR2. No enrichment was found for the previously described KDR+PDGFR-α+ progenitors. MESP1-mCherry derivatives contained an enriched percentage of NKX2-5-eGFP and Troponin T expressing cells, indicating preferential cardiac differentiation; this was enhanced by inhibition of the Wnt-pathway. Furthermore, MESP1-mCherry derivatives harboured smooth muscle cells and endothelial cells, demonstrating their cardiac and vascular differentiation potential under appropriate conditions. The MESP1-NKX2-5 hESC reporter line allows us to identify molecular cues crucial for specification and expansion of human cardiac mesoderm and early progenitors and their differentiation to specific cardiovascular derivatives.

INTRODUCTION

Human pluripotent stem cells (hPSCs) offer exceptional opportunities for regenerative medicine, drug discovery and toxicity, disease modelling and developmental biology because of their ability to differentiate in principle into any cell type of the human body. In particular, their differentiation towards cell types that are challenging to obtain and maintain as primary cultures from tissues with limited or no self-repair capacity, such as the brain and heart, have made them attractive as research models. Previously, we and others have shown that cardiomyogenesis of hPSCs *in vitro* recapitulates signalling events and sequential transcriptional activation that occurs during heart development *in vivo* [1, 2]. Moreover, as knowledge of the regulatory mechanisms that govern *in vivo* cardiac development has advanced, it has been possible to translate this to differentiation protocols based on stepwise temporal modification of signalling pathways. This has led to a remarkable increase in the yield of cardiac cells not only from hESCs but also from human induced pluripotent stem cells (reviewed in [3]).

However, despite these advances, the cardiac derivatives obtained contain heterogeneous populations of cardiac cells: not only different subtypes of cardiomyocytes but also endothelial cells and smooth muscle cells. This is because the molecular cues underlying specification of early human cardiac progenitors towards specific cardiac subtypes have not been fully elucidated. For future clinical and pharmaceutical applications, better insight into the differentiation of cardiac cell subtypes is necessary.

Several studies have shown that different cardiac subtypes are derived from multiple early cardiac progenitors that segregate soon after gastrulation[4-6]. The molecular mechanisms that play a role in the formation of early cardiac progenitors however, remain largely unknown[4, 7]. In order to study the onset of cardiac differentiation in human, it is necessary to identify early populations from common progenitors at the mesodermal stage of development and identify the molecular pathways involved in the further differentiation of the cardiac lineage.

Mesoderm transcription factor MESP1 is important in this context as *in vivo* lineage tracing in mouse has demonstrated that almost all cells of the heart are derived from a common MESP1-expressing mesodermal progenitor, including myo-, endo, and epicardium, and cells of the conduction system[8-10]. MESP1 is transiently expressed in the anterior mesoderm from the onset of gastrulation prior to cardiac crescent formation[8, 11, 12]. Furthermore, MESP1 overexpression in mouse ESCs showed that MESP1 induces the expression of cardiovascular genes[13-15]. A recent study in mouse illustrated that besides the cardiac lineage, MESP1 derivatives also encompass cells of the hematopoietic- and skeletal myogenic lineages, which emerge depending on the endogenous and exogenous cues present during MESP1 expression[16]. Of note, all these studies have been performed in mice. However, to instigate advances in human heart development, regenerative medicine, disease modelling and drug testing, it is necessary to understand the earliest steps of cardiac differentiation in a human model.

In order to identify, isolate and characterise MESP1 expressing cells during human cardiomyogenesis, we introduced the mCherry reporter into the MESP1 locus of the NKX2-5eGFP hESC reporter line generated previously [17], leading to a dual reporter system for these early cardiovascular lineage markers in hESCs. We were able to isolate the MESP1-expressing mesoderm progenitors in a cardiac-inducing environment and demonstrate their ability to differentiate further to a highly enriched NKX2-5eGFP expressing cardiac population. We further confirmed the presence of other mesoderm and cardiac progenitor populations in MESP1-mCherry expressing-derivatives, including CD13+ROR-2+ and PDGFR-α+KDR+ populations [18-20]. Although CD13, ROR-2, and PDGFR-α expression were strongly enriched, they were not specifically expressed in the MESP1-mCherry derivatives. In addition, no enrichment was found for the previously described PDGFR-α+KDR+ population. Finally, we demonstrate that this MESP1^{mCherry}-NKX2-5^{eGFP} dual reporter hESC line is a useful tool to study the differentiation of pre-cardiac mesoderm towards early cardiac

progenitor cells and their derivatives. It now provides an opportunity to study the molecular mechanisms underlying the onset of progenitor subtypes formation specifically in the cardiac lineage in human. This hESC dual reporter will contribute to providing better insights into cardiomyocyte determination and form the basis for improved differentiation protocols for specifying cardiac subtypes (atrial, ventricular, pacemaker cells). This will be relevant to future pharmaceutical and clinical applications of specific cardiac cell subtypes.

METHODS

MESP1 Gene Targeting in hESCs

To generate the *MESP1* targeting vector, the bacterial artificial chromosome (BAC) RP11-975L13 (Source Bioscience, Lifesciences) was modified by recombineering (Gene Bridges). The mCherry cassette and a selection marker replaced the MESP1 exon 1 sequence (oligonucleotides used for recombineering: MESP1_5arm_PvuI and MESP1_3_arm, **Supp. Table S1**). The modified *MESP1* locus and the surrounding 5'and 3'homology arms were subsequently subcloned into the *MESP1* targeting vector. The final vector comprised a 8.6-kilobase (kb) 5'homology arm, mCherry, *loxP*-flanked G418 resistance cassette and a 2.3-kb 3'homology arm.

The targeting vector was linearized by PvuI digestion and 20 µg was electroporated into 9x106 NKX2-5-eGFP reporter hESCs (HES3-NKX2-5$^{eGFP/w}$)[17] as described previously[21]. Targeted clones were identified by PCR using oligonucleotides specific for mCherry (P1) and a region immediately 3'of the targeting vector (P2) (**Fig. S1, Table S1**).

Targeted clones were clonally derived and transfected with Cre-recombinase to excise the *loxP*-flanked neomycin-resistance cassette as described previously[22]. Neomycin excision was confirmed by PCR (**Fig. S1, Table S1**). Undifferentiated, targeted clones were fixed in 2% paraformaldehyde and were used for immunocytochemistry (Nanog, Tra-181, and Oct-4) to confirm their pluripotent status. For karyotyping, 20 metaphase chromosome spreads were examined for each line.

Single Cell Sorting

For single cell sorting, hESCs were enzymatically passaged using 1x TrypLE Select (Life Technologies). After dissociation, cells were resuspended in FACS sorting buffer (PBS with 0.5% BSA and 2.5mM EDTA) and filtered through a 40-µm cell strainer (Falcon). Cells were sorted using a BD ARIA III flow cytometer into 96 wells flat-bottom low attachment plates, live cells were gated on the basis of side scatter, forward scatter and propidium iodide exclusion. Flow cytometric gates were set using control cells labelled with the appropriate isotype control antibody.

hESC Culture and Cardiac Differentiation

MESP1$^{mCherry/w}$ NKX2-5$^{eGFP/w}$ hESCs were cultured on mouse embryonic fibroblasts on 2.85 cm2 organ dishes (Falcon), in 1 mL hESC medium (DMEM F12, Non Essential Amino Acids, Knock Out Serum (Gibco by Life Technologies); bFGF (Miltenyi Biotec)) and were mechanically passaged[23]. One or two days before differentiation, cells were passaged onto a 12-well tissue culture dish coated with Matrigel (BD Biosciences; growth factor reduced, phenol red–free), in 1 mL of hESC medium per well. On the first day of differentiation, day 0, hESC medium was replaced by the low insulin serum-free medium BPEL (BSA, polyvinyl alcohol, essential lipids, as previously described)[17, 24], containing the cytokines BMP4 (30 ng ml^{-1}, R&D Systems) and Activin A (30 ng ml^{-1}, Miltenyi Biotec), and Chir99021 (1.5uM, Axon Medchem). At day 3 of differentiation, the medium was refreshed with BPEL containing 5uM Xav939 (R&D). On day 7 of differentiation, the medium was replaced with BPEL without growth factors.

Isolation and Re-aggregation of MESP1-mCherry cells

On day 3 of differentiation, cells were sorted by flow cytometry as described above, based on mCherry expression (both positive and negative populations). Flow cytometric gates were set using undifferentiated hESCs. Sorted cells were seeded in a 96-well V-shaped non-adherent plate (Greiner Bio One) at 20,000 cells per well in 50 µL BPEL in the presence of Xav939 (5uM, R&D) to enhance cardiac differentiation. Aggregation was initiated by a brief centrifugation step for 3 minutes at 480g. Four days after replating, aggregates were refreshed with BPEL without addition of growth factors or inhibitors.

Analysis of MESP1-mCherry Derivatives

Cardiomyocytes derived from MESP1-mCherry-sorted cells were analyzed by flow cytometry and immunohistochemistry (IHC) on day 3, 5 and day 14 of differentiation (0, 2 and 11 days after replating). For IHC, MESP1-mCherry-derived aggregates were fixed in 2% PFA at day 14. For alpha-actinin and SM-HC IHC, MESP1-mCherry-derived aggregates were dissociated using 1x TrypLE Select, plated on matrigel in BPEL, and fixed after four days.

For flow cytometric analysis, aggregates were collected, dissociated as described before, and resuspended in FACS washing buffer (PBS with 2% FCS and 2.5mM EDTA). For analysis of intracellular markers, cells were treated with FIX & PERM® Cell Fixation and Permeabilization Kit (Life Technologies). Analyses were carried out using a MACS-Quant VYB flow cytometer (Miltenyi Biotec) and FlowJo Software. Antibodies are listed in Supplementary Table S4 (**Table S4**). Specimens were analysed with confocal laser microscope (Leica TCS SP8). Images were acquired with Leica LAS AF software. MESP1-mCherry expressing cells were visualized in monolayer differentiation at day 3, using a Nuance™ Multispectral Imaging System.

RNA isolation, Quantitative Real-time PCR and Microarray Analysis

Total RNA was isolated using either the RNeasy micro (Qiagen) or Nucleospin RNA XS kits (Macherey Nagel) according to manufacturer's protocols, and treated with RNase-free DNase. Between 100 ng to 1 ug RNA was reverse transcribed into cDNA with I-scriptTM

cDNA synthesis kit (Bio-rad), according to manufacturer's protocol. Real-time quantitative PCR was performed on a CFX96 Real Time System (Biorad). All experiments were carried out in triplicate with iTaq™ Universal SYBR® Green Supermix. Expression levels were normalised to the housekeeping gene hARP and multiplied by 1000 for the purpose of presentation. Primer sequences are listed in Supplementary Table S2 (**Table S2**). Microarray analysis was performed on three biologically independent replicates using Illumina human HT-12v4 arrays (ServiceXS). Illumina microarray data were processed using the lumi package and differentially expressed genes were identified using the limma package, included in R/Bioconductor[25, 26]. ConsensusPathDB-human (http://cpdb.molgen.mpg.de/) web server was used for gene ontology analysis. Additional analysis was performed using Genespring (Agilent Technologies).

RESULTS

Generation of a MESP1mCherry-NKX2-5eGFP dual cardiac reporter

To identify, purify and characterize human pre-cardiac mesodermal progenitors and study their further differentiation, we generated a dual fluorescent hESC reporter line by inserting the sequence encoding mCherry into the MESP1 locus of an existing NKX2-5^{eGFP} reporter line by homologous recombination **(Fig 1a, b)**. A PCR screening strategy was performed to identify correctly targeted clones **(Fig S1a,b)**. Twenty correctly targeted clones were obtained (11% targeting efficiency) from which four clones showed the loxP-flanked neomycin-resistance cassette was excised following transfection with Cre recombinase (not shown) [22]. We proceeded with clone 2.20B3 **(Fig. S1c)**. hESCs from clone 2.20B3 displayed a high immunoreactivity for NANOG, TRA-181, OCT-4, and EPCAM antibodies, confirming no loss of pluripotency during targeting and selection procedures **(Fig S2a,b)**. Furthermore, chromosome analysis demonstrated no abnormal karyotypes in these targeted lines **(Fig S2c)**.

To demonstrate that the targeted MESP1$^{Mcherry/w}$-NKX2-5$^{eGFP/w}$ hESC lines faithfully recapitulated MESP1 activity, hESCs were differentiated as monolayers to cardiac cells by addition of the growth factors BMP4 and Activin-A, as well as GSK-3-β inhibitor Chir99021 from day 0 to day 3, followed by the Wnt-signalling antagonist pathway inhibitor Xav939 from day 3 to day 7 of differentiation **(Fig 1c)**. Red fluorescence of mCherry was clearly visible in a subset of cells at day 3 of differentiation **(Fig 1d)**. Cells expressing mCherry appeared to have undergone EMT, based on their mesenchymal-like morphology.

MESP1mCherry-NKX2-5eGFP hESCs differentiate towards mesoderm and the cardiac lineage

Quantitative PCR (qPCR) analysis of the targeted cell line during differentiation towards the cardiac lineage confirmed transient expression of mCherry, peaking at day 3 of differentiation and mirroring endogenous *MESP1* expression **(Fig 2a)**. In agreement with previous *in vitro* and *in vivo* studies, expression levels of mesendodermal genes *Brachyury (BRY)* and *Eomesodermin (EOMES)* displayed peak values at day 2 of differentiation, before that of *MESP1*. Expression levels of *NKX2-5* were clearly detectable from day 9 of differentiation, just before the appearance of contractile areas. Expression of *NKX2-5* further increased at day 12, confirming the ability of the MESP1^{mCherry}-NKX2-5^{GFP} reporter line to differentiate towards the cardiac lineage. Flow cytometric analysis showed that MESP1-mCherry expressing cells first appeared around day 2, peaking at day 4 (8.0% ± 0.5% (mean ± SEM)), and becoming negligible by differentiation day 8 **(Fig 2b,c)**, reflecting the gene expression profile. NKX2-5-eGFP protein was detected later, first appearing on day 8 and continuing to increase to day 14 of differentiation (28.9% ± 1.7%), reflecting our previous findings[17]. These results demonstrated that the MESP1^{mCherry} and the NKX2-5^{eGFP} reporters allowed visualization of early MESP1-expressing progenitors, followed by the formation of NKX2-5-expressing cardiac cells during hESC differentiation.

Gene Expression Profiling of MESP1-mCherry progenitors

To further characterize the MESP1-mCherry progenitors, we isolated MESP1-mCherry positive cells (MESP1-mCherry pos) at day 3 of differentiation and compared their gene expression profile with MESP1-mCherry negative cells (MESP1-mCherry neg) and undifferentiated hESCs of the same line *(Fig 3a)*. A correlation heatmap between samples is shown in supplemental figure 3 *(Fig. S3)*. In three biological replicates, 722 genes showed an increase in expression of ≥ 1.5 fold when compared to MESP1-mCherry neg cells **(Fig 3b)**. In agreement with their mesodermal character, MESP1-mCherry pos cell fraction was enriched for EMT- and mesendodermal related genes, including *MESP1* itself, *SNAI1, SNAI2, MSX1, FOXC1, GSC, MIXL1, EOMES, LHX1, FOXF1, and IRX3.* Although the differences were greater in MESP1-mCherry pos cells, mesendodermal and EMT-related gene levels were higher in both MESP1-mCherry pos and neg fractions compared to undifferentiated hESCs, indicating that cells had undergone some form of mesoderm differentiation in the protocol used. Furthermore, the enrichment of N-cadherin (*CDH-2*) and vimentin (*VIM*), and decrease of E-cadherin (*CDH-1*) and *EPCAM* are also strongly associated with EMT. As expected, transcripts of pluripotency genes, including *SOX2, OCT3-4,* and *NANOG*, were significantly decreased. Furthermore, early cardiac genes, such as *GATA4, TBX3, MYL4, GATA6,* and *HAND1*, were enriched indicating the pre-cardiac character of MESP1-mCherry progenitors. In addition, genes encoding extracellular matrix proteins were increased, including several collagens, integrins, laminins, and fibronectin, as well as extracellular matrix (ECM)-receptors, such as integrin α-5 (*ITGA5*). These proteins could play an important role in stage-dependent processes such as mesoderm migration or niche formation of the pre-cardiac mesodermal cell population.

The microarray data also indicated the expression of genes of various signalling pathways in the MESP1-mCherry pos population, including Notch ligand *DLL3, BMP4, FGF17*, the BMP-antagonist *CER1*, Wnt inhibitors *DKK1* and *DKK4*, and other components of (non)

canonical Wnt signalling, such as the ligand Wnt5a and (co)-receptors *FZD2*, *ROR-2*, and *RSPO3*. Protein complex analysis showed 100% enrichment of the non-canonical WNT5a/FZD2/ROR-2 complex (data not shown). In agreement with this, pathway analysis showed significant enrichment (P-value ≤ 0.01) in extracellular matrix organization, Wnt signalling pathway, cardiac progenitor differentiation, and heart development **(Fig 3c)**. qPCR analysis validated enhanced expression of a selection of the genes upregulated in the microarray data **(Fig 3d)**.

Surface Marker Expression Analysis of MESP1-mCherry progenitors

Further microarray analysis identified several genes encoding mesoderm associated cell surface proteins, including *NCAM1, ROR-2, CD13, CDH-2, CDH-11,* and *PDGFR-α*, which showed increased expression in MESP1-mCherry pos cells (Fig 3a). FACS analyses showed that large proportions of the MESP1-mCherry pos cells expressed cell surface proteins PDGFR-α (approximately 70%), CD13 (approximately 97%), and ROR-2 (approximately 96%) **(Fig 4a)**. However, none of these proteins marked the MESP1-mCherry pos population exclusively, since expression was also clearly detected in the MESP1-mCherry neg cell populations **(Fig 4b)**. Further, only a small percentage in both the MESP1-mCherry pos and neg population was co-expressing PDGFR-α and KDR, in agreement with the microarray analysis, which showed that KDR was not differentially expressed in the MESP1-mCherry pos population.

Generation of Cardiac Cells from Isolated MESP1-mCherry progenitors

To investigate whether MESP1-mCherry pos progenitors were able to differentiate towards the cardiac lineage, MESP1-mCherry pos cells were isolated on day 3 of differentiation, seeded as aggregates or in monolayer in the presence of Wnt-pathway agonist Xav939 until differentiation day 7, and maintained until differentiation day 14 **(Fig 5a)**. To evaluate how mesoderm progenitor populations are represented in MESP1-mCherry sorted derivatives, we initially performed FACS analysis on differentiation day 5 (2 days after replating). ROR-2+CD13+ cells were slightly enriched in the MESP1-mCherry pos population, compared to the MESP1-mCherry neg population; approximately 73% ± 9% (mean ± SEM) vs. 39% ± 16% respectively **(Fig 5b)**. Furthermore, almost all MESP1-mCherry derivatives on day 5 expressed PDGFR-α (approximately 98% ± 1% (mean ± SEM)), while only 50% ± 4% of the day 5 MESP1-mCherry neg derivatives did. Interestingly, there was no enrichment of a KDR+PDGFR-α+ cardiac progenitor population in the MESP1-mCherry pos population, compared to the MESP1-mCherry neg derivatives (approximately 22% ± 2% (mean ± SEM) vs. 28% ± 12%, respectively) **(Fig 5b)**. Similar results were found at other time points during differentiation (day 4-8) **(Fig. S4)**.

On day 8 and day 14 of differentiation, the expression of several cardiac-associated genes was enriched in the MESP1-mCherry pos derivatives compared to the MESP1-mCherry neg derivatives, including cardiac genes *NKX2-5* and *ISL1*, smooth muscle cell markers *SMA* and *MYH11*, and endothelial marker *PECAM-1* **(Fig 5c,d)**. Additionally, NKX2-5 expression, as determined by flow cytometric quantification of eGFP expression, was significantly enriched in the MESP1-mCherry pos population, treated with Xav939 (80% ± 4% (mean ± SEM)) when compared to MESP1-mCherry neg cells under similar differentiation conditions (18% ± 6%) **(Fig 6a,b,c)**. The eGFP+ cells detected in the MESP1-mCherry neg day 14 aggregates may have been derived from a small percentage of MESP1-mCherry pos cells that subsequently appeared in the MESP1-mCherry neg population following sorting. Indeed, low expression levels of mCherry and MESP1 could be detected in negative sorted samples at day 3 and day 4 (one day after reaggregation) **(Fig. S5)**.

The importance of inhibiting Wnt/β-catenin signalling following mesoderm induction to generate NKX2-5-expressing cardiac progenitors was highlighted by the low levels of eGFP in the untreated MESP1-mCherry pos (23% ± 4%) and MESP1-mCherry neg derivatives (0.50% ± 0.4%). Furthermore, in all conditions, the NKX2-5^{eGFP}+ derivatives were spontaneously contracting on differentiation day 14 (data not shown).

To determine whether other specific signalling pathways could affect cardiac differentiation from mesoderm, three other major pathways associated with cardiac differentiation were inhibited after MESP1-mCherry sorting and re-aggregation (from day 3 to day 7). Inhibitors of these pathways included TGF-β/act/Nodal antagonist SB431542, and BMP inhibitor Dorsomorphin. None of these inhibitors led to an increase in GFP expression at day 14 when compared to control treatment. In fact, GFP expression showed a tendency to decrease in the SB431542-treated cells **(Fig S6)**.

Day 14 MESP1-mCherry pos derivatives treated with Xav939, expressed various cardiac lineage markers, including α-Actinin (cardiomyocytes), PECAM-1 (endothelial cells), and SMA and SMC-myosin (expressed in immature cardiomyocytes as well as smooth muscle), indicating the MESP1-mCherry pos cell population can generate all three different cell types of the cardiac lineage **(Fig 6d)**. In addition, flow cytometric analysis showed enrichment of cTNT (approximately 51% ± 9% (mean ± SEM)) and PECAM-1 (approximately 2.2% ± 0.2% (mean ± SEM)) expressing cells in day 14 MESP1-mCherry pos derivatives, compared to the MESP1-mCherry neg derivatives (approximately 11% ± 5% and 0.62% ± 0.1% respectively) **(Fig 6e)**.

SIRPA+ VCAM1+ co-expression marks NKX2-5-eGFP+ cardiomyocytes

In addition to the characterization and differentiation of MESP1^{mCherry} progenitors and their further differentiation to NKX2-5eGFP cardiac cells, we studied whether the previous described cell surface proteins SIRPA and VCAM1 are specifically marking hESC-derived-cardiomyocytes[27, 28]. We analyzed SIRPA-VCAM1 and cTnT expression on cardiac cells at day 14 of differentiation of NKX2.5-eGFP-HESCs **(Fig S7)**. We showed that the SIRPA+VCAM1+ double positive population was highly enriched for NKX2-5-eGFP+ cells **(Fig S7a)**. In addition, the vast majority of NKX2-5-eGFP+ cells were cTnT+ **(Fig S7b)**. Similar results were described in a recent study by Skelton et al., in which SIRPA+VCAM1+NKX2-5-eGFP+ cardiomyocytes were shown to arise from a SIRPA+NKX2-5-eGFP+ intermediate progenitor population[28].

DISCUSSION

Genetically modified reporter lines are proving increasingly valuable for developing precise protocols for the directed differentiation of various lineages from human PSCs[29-32]. Here we increased the degree of complexity of the processes that can be analysed by creating a double transgenic reporter hESC line, taking advantage of endogenous activation of the key cardiac transcription factors *MESP1* and *NKX2-5*[17]. We introduced the fluorescent protein mCherry into the genomic locus of the mesodermal transcription factor MESP1, in *NKX2-5*^{eGFP} hESCs. As predicted from mouse developmental biology studies, the *MESP1*^{mCherry}-*NKX2-5*^{eGFP} line firstly expressed mCherry under conditions of mesoderm induction, and later GFP+ cardiac progenitors and cardiomyocytes emerged in the cultures, demonstrating the value of hESCs as *in vitro* model of previously inaccessible stages of early human development.

MESP1-mCherry pos cells were first detected on day 2 of differentiation with peak levels between day 3 and 4, which is in agreement with our earlier observations during cardiac differentiation of hESCs[1]. We demonstrated that sorted and reaggregated MESP1-mCherry pos cells on differentiation day 3 have a much higher potential to differentiate to cardiovascular derivatives than MESP1-mCherry neg cells, as demonstrated by (i) the appearance of functional beating cardiomyocytes and (ii) high numbers of *NKX2-5*^{eGFP}+ cardiac cells in the population on day 14 of differentiation (11 days after sorting). At the same stage of differentiation, enriched gene and protein expression were found for PECAM-1, SMA, and cTNT, suggesting that MESP1-mCherry cells can differentiate to endothelial cells, smooth muscle cells and cardiomyocytes, respectively.

Microarray analysis of MESP1-mCherry pos and MESP1-mCherry neg cells revealed new insights into the transcriptional profile of human pre-cardiac mesodermal progenitors. In particular, transcription factors and signalling pathways associated with mesoderm formation, EMT, early cardiac development and components of the ECM were highly enriched in MESP1-mCherry pos cells. This is broadly in accordance with the molecular signature of MESP1-GFP expressing mouse cells[33]. However, disparities possibly related to interspecies differences, differentiation protocols, or technical variations (i.e. random integration of an isolated regulatory sequence of the *Mesp1* mouse promoter versus homologous recombination in the human *MESP1* locus) were evident. For example, the expression of cardiac transcription factors such as *HAND2, MEIS2, SMARCD3, HEY1, HEY2,* and *TBX2* was increased in MESP1-GFP positive cells, whereas in our study these genes were not enriched in the MESP1-mCherry pos population. However, expression of these genes was increased at later stages following cardiac differentiation of sorted cells, suggesting that human MESP1-mCherry pos cells may be a more restricted mesodermal population.

Transition of undifferentiated epithelial pluripotent stem cells to mesodermal cells requires the activation of EMT-associated genes. Indeed, MESP1-mCherry pos cells exhibit an obvious increase in levels of EMT-related transcription factors SNAI1 and SNAI2. In agreement, increased expression of other EMT-related genes, including Vimentin and CDH-2, and decreased levels of epithelial proteins, such as CDH-1 and EPCAM, suggested that EMT took place in MESP1-mCherry pos cells. In addition, enrichment of many ECM-related transcripts, such as those for collagens, integrins, fibrillin, matrix remodellers, and receptors, like the Apelin Receptor (APLNR), suggested formation of an appropriate niche for MESP1-expressing cells [34, 35]. Alterations in cell-cell and cell-matrix interactions are crucial for biological processes such as EMT, intracellular interactions, cell-fate decisions, or migration of pre-cardiac MESP1-progenitors from the anterior part of the primitive streak to form the bilaterally situated cardiac crescent *in vivo* [36-39]. Further study is required to identify the role of specific ECM components and receptors for expansion and further differentiation of MESP1+ cells.

Several signalling pathways were also significantly activated in MESP1-mCherry pos cells. In particular, components of the Wnt signalling pathway were affected, including the Wnt-inhibitors DKK1 and DKK4, the (non)canonical pathway ligands WNT4 and WNT5a, and the receptors ROR-2 and FZD2. Previous studies have indicated an important role for activated canonical Wnt-signalling during hESC to mesendoderm differentiation[40-43]. Subsequent inhibition of this pathway by DKK-1 resulted in improved cardiac differentiation, confirming the need to inhibit canonical Wnt-signalling following mesoderm formation for efficient cardiac differentiation[14, 40]. Conversely, at the same stage, activated non-canonical signalling has been associated with driving cardiac differentiation[44]. Non-canonical Wnt signalling has been linked with different molecular mechanisms, including canonical Wnt and/or β-catenin signalling inhibition[42, 45, 46], progenitor proliferation[47], and cellular movements and polarity. Here, we confirmed the importance of Wnt-inhibition in mesoderm to cardiac progression, as we found a major increase in the percentage of NKX2-5^{eGFP} expressing cells upon treatment with the chemical Wnt signalling antagonist, Xav939. Other pathways that have been shown to play crucial roles in the early stages of mesoderm formation, such as the BMP, Notch and FGF pathways, were also present in the MESP1-mCherry pos population, suggesting cross-talk of components of these pathways during these early pre-cardiac mesoderm stages[41].

We also examined expression of cell surface markers of mesodermal and cardiovascular progenitors during the differentiation of *MESP1*^{mCherry}-*NKX2-5*^{eGFP} cells. Several studies have reported that PDGFR-α and Flk1/KDR are expressed on early cardiovascular progenitor cells, giving rise to cardiomyocytes, endothelial cells, and vascular smooth muscle cells[5, 19, 20]. In recent studies using hESCs, an early mesoderm population co-expressing CD13 (ANPEP) and ROR-2 has been identified [18, 48]. This population consists of a KDR+PDGFR-α+ subpopulation, which has the potential to give rise to cardiovascular cells. In agreement, we demonstrated a significant increase in CD13, ROR-2, and PDGFR-α on day 3 MESP1-mCherry pos cells and on day 5 derivatives. However, these surface markers were also expressed in a high percentage of the MESP1-mCherry neg populations, indicating that they mark a broader population than only pre-cardiac mesoderm[27]. Interestingly, in MESP1-mCherry pos derivatives we did not find enrichment of KDR+ cells. These findings indicated that,

besides the previously described Flk1/KDR+ PDGFR-α+ cardiac progenitor population, other MESP1-derived pre-cardiac mesoderm progenitor populations exist, that (co)express other (cell surface) markers, and have the potential to differentiate towards *NKX2-5*^{eGFP} expressing cardiac cells. In previous studies, the cardiac potential of other subpopulations besides the Flk1/KDR+ PDGFR-α+, was not tested[18, 20].

One of the major challenges in cardiovascular research is cardiac regeneration and improved cardiac function in heart failure patients. Tissue engineering, cellular therapy, endogenous activation of cardiac progenitors cells, or a combination of these approaches may lead to novel therapeutic strategies[49]. However, a key concern is the selection of the right cell type for transplantation or enhanced *in situ* proliferation, as this will be important for survival and engraftment, for safety (risk of tumor formation), and for the ability to form the required cell type(s) [50, 51]. A recent study showed the ability of a hESC-derived CD13+ROR-2+KDR+PDGFR-α+ cardiac progenitor population to migrate, couple, and form cardiac cells after being transplanted into ex vivo human fetal ventricular heart tissue, without formation of teratomas[18]. As pre-cardiac MESP1-progenitors are able to form almost all cell types of the heart, it will be of interest to examine both the developmental and regenerative capacity of these cells upon transplantation into both healthy and damaged hearts. Even though many hurdles have to be overcome, transplantation studies using cardiac-lineage-restricted progenitors may shed more light on future heart regeneration therapies. In addition, understanding the molecular cues that are important for expansion of mesodermal cells and their specific differentiation to the cardiac lineage may lead to novel therapies which are based on the progression of endogenous cardiac progenitor cells to functional specialized cardiovascular cells.

CONCLUSION

We conclude that the MESP1$^{mCherry/w}$-NKX2-5$^{eGFP/w}$ dual cardiac fluorescent reporter hESC line in this study is a useful tool for dissecting the molecular and cellular mechanisms related to the earliest steps in cardiac development in live cells. This is novel way to carry out fate analysis of this subpopulation. Purified mesodermal MESP1-mCherry pos cells efficiently differentiated to beating NKX2-5^{GFP}+ cells by inhibition of the Wnt-pathway. Besides a better understanding of underlying mechanisms, this human cardiac dual reporter line will make it feasible to identify and specify culture conditions for expansion of a mesodermal cell population and their further differentiation towards cardiac progenitor cells and specific subtypes of the cardiac lineage *in vitro*. This is of particular interest for future studies on cardiac toxicity, drug screening, tissue engineering, and regenerative medicine.

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FIGURES

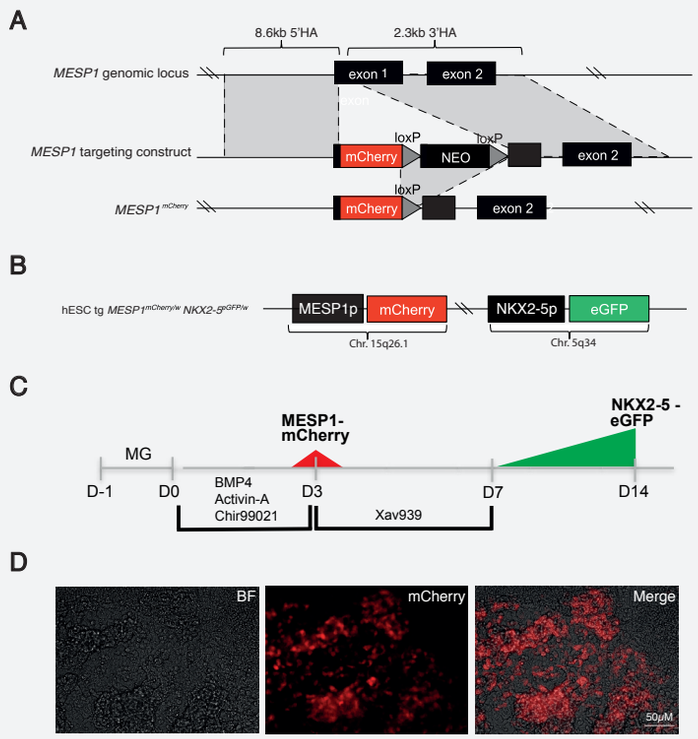

Figure 1. Generation of a MESP1mCherry-NKX2-5eGFP dual reporter hESC line. **A:** Schematic overview of wild-type MESP1 allele, MESP1 targeting construct, and targeted MESP1 allele. **B:** Schematic overview of the dual cardiac fluorescent reporter line. p: endogenous gene regulatory elements of either MESP1 (MESP1p) or NKX2-5 (NKX2-5p). **C:** Cardiac monolayer differentiation protocol in BPEL. MG: matrigel. Tg: transgenic. **D:** MESP1-mCherry expressing cells at day 3 of cardiac differentiation, visualized by a Nuance™ Multispectral Imaging System.

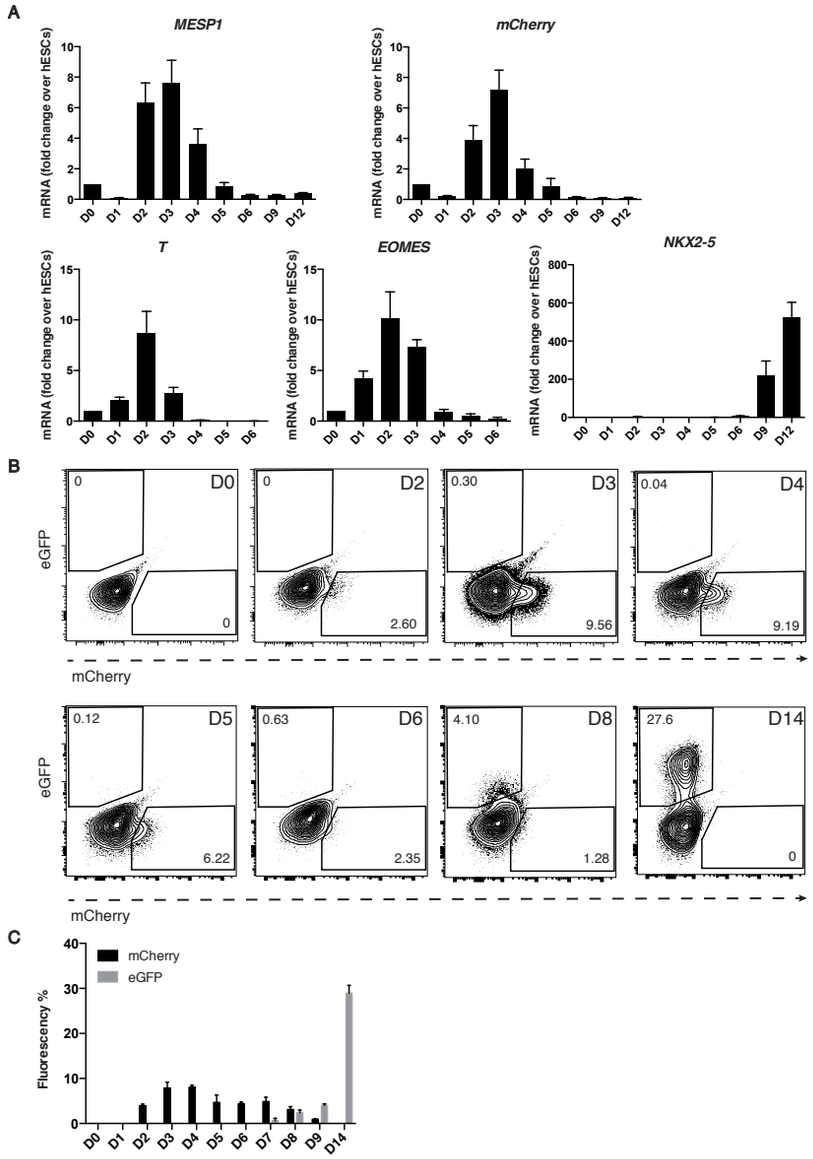

Figure 2. Timecourse of MESP1mCherry-NKX2-5eGFP hESCs differentiating towards the cardiac lineage. A: mRNA levels of mesendodermal genes *BRACHYURY T* (T) and *EOMESODERMIN (EOMES)*, pre-cardiac mesoderm gene *MESP1, mCherry* reporter gene, and early cardiac gene *NKX2-5* during monolayer differentiation. Results are normalized for the transcript expression at day 0 (undifferentiated hESCs) (n=3, error bars indicate SEM). **B:** Percentages of mCherry and eGFP protein expression upon monolayer differentiation. C: Quantification of percentages of mCherry and eGFP expressing cells during monolayer differentiation (n= 4-8; error bars indicate SEM).

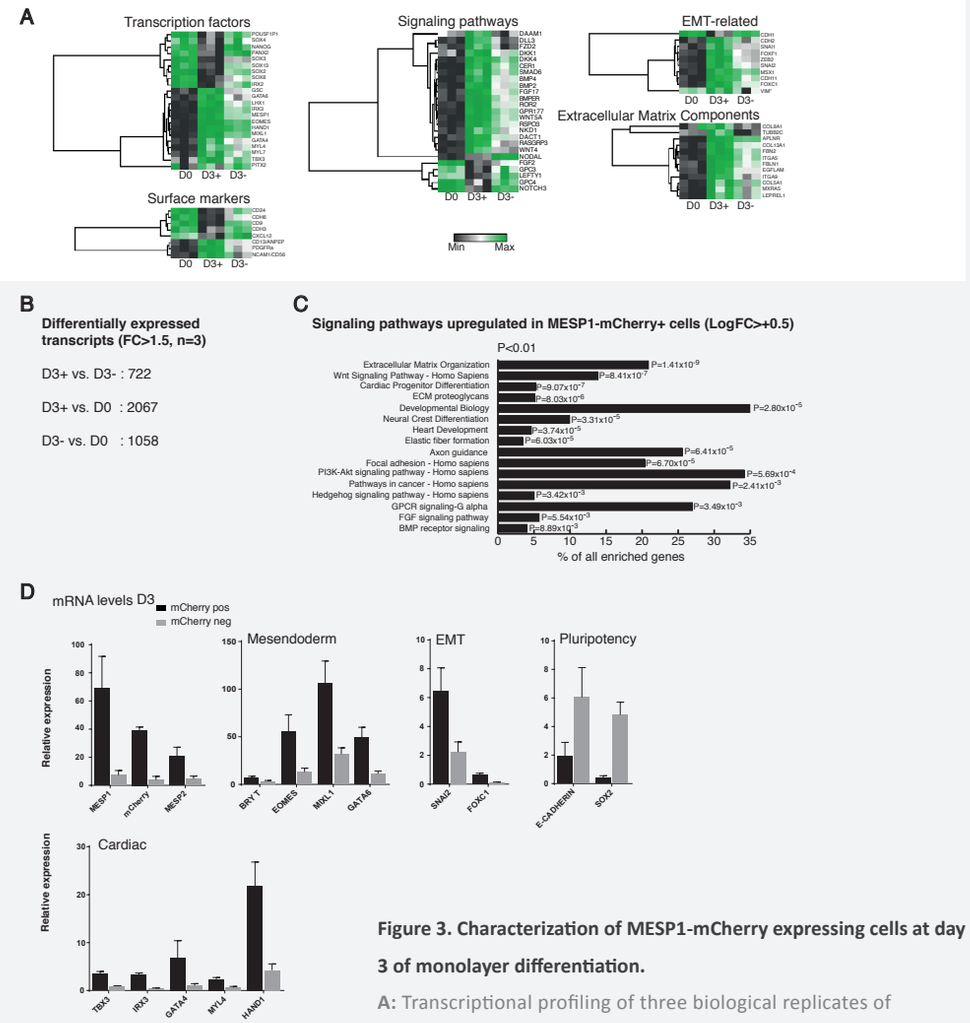

Figure 3. Characterization of MESP1-mCherry expressing cells at day 3 of monolayer differentiation.

A: Transcriptional profiling of three biological replicates of undifferentiated hESCs (D0), differentiation day 3 MESP1-mCherry pos cells (D3+), and MESP1-mCherry neg cells (D3-). D3+ samples were enriched for specific transcription factors, cell surface markers, ECM components, EMT-related genes, and signalling pathway ligands and receptors. Clustering of the samples is based on hierarchical clustering of microarray gene expression profiling. Shown are a selection of transcripts expressed in D3+ with a fold change ≥ 1.5 compared to D3- for three independent paired experiments (n=3).* indicates differences in only two independent experiments. GEO accession number: GSE56721. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56721 **B:** Number of transcripts that was ≥ 1.5 fold differentially expressed (n=3) between D3+ and D0, D3- and D0, and D3+ and D3- samples. **C:** Signalling pathway analysis of enriched genes in MESP1-mCherry expressing cells. P<0.01 for selected pathways. Percentages are based on all enriched genes. **D:** Validating microarray results by qRT-PCR analysis of selected transcripts in purified MESP1-mCherry pos and MESP1-mCherry neg cell populations at differentiation day 3 (n=3, error bars indicate SEM). Values are normalized for hARP levels.

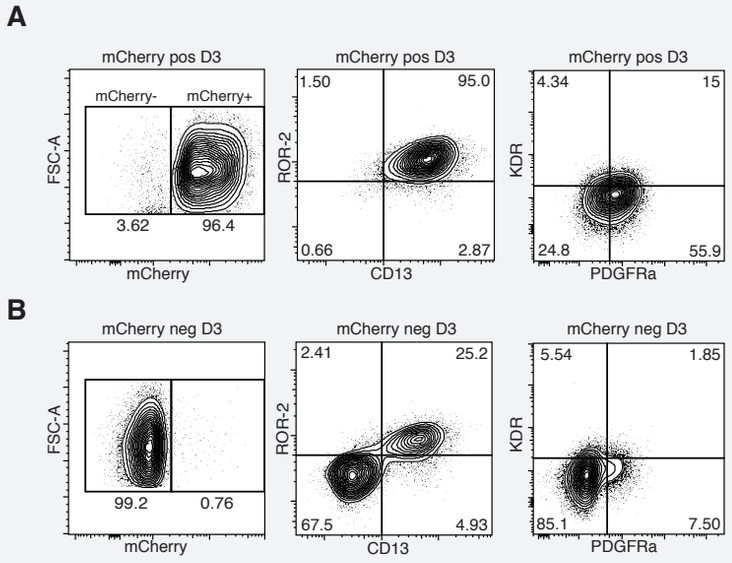

Figure 4. Flow cytometry analysis of cell surface markers CD13, ROR-2, KDR, and PDGFR-α on **(A)** MESP1-mCherry pos and **(B)** MESP1-mCherry neg sorted populations at day 3 of differentiation.

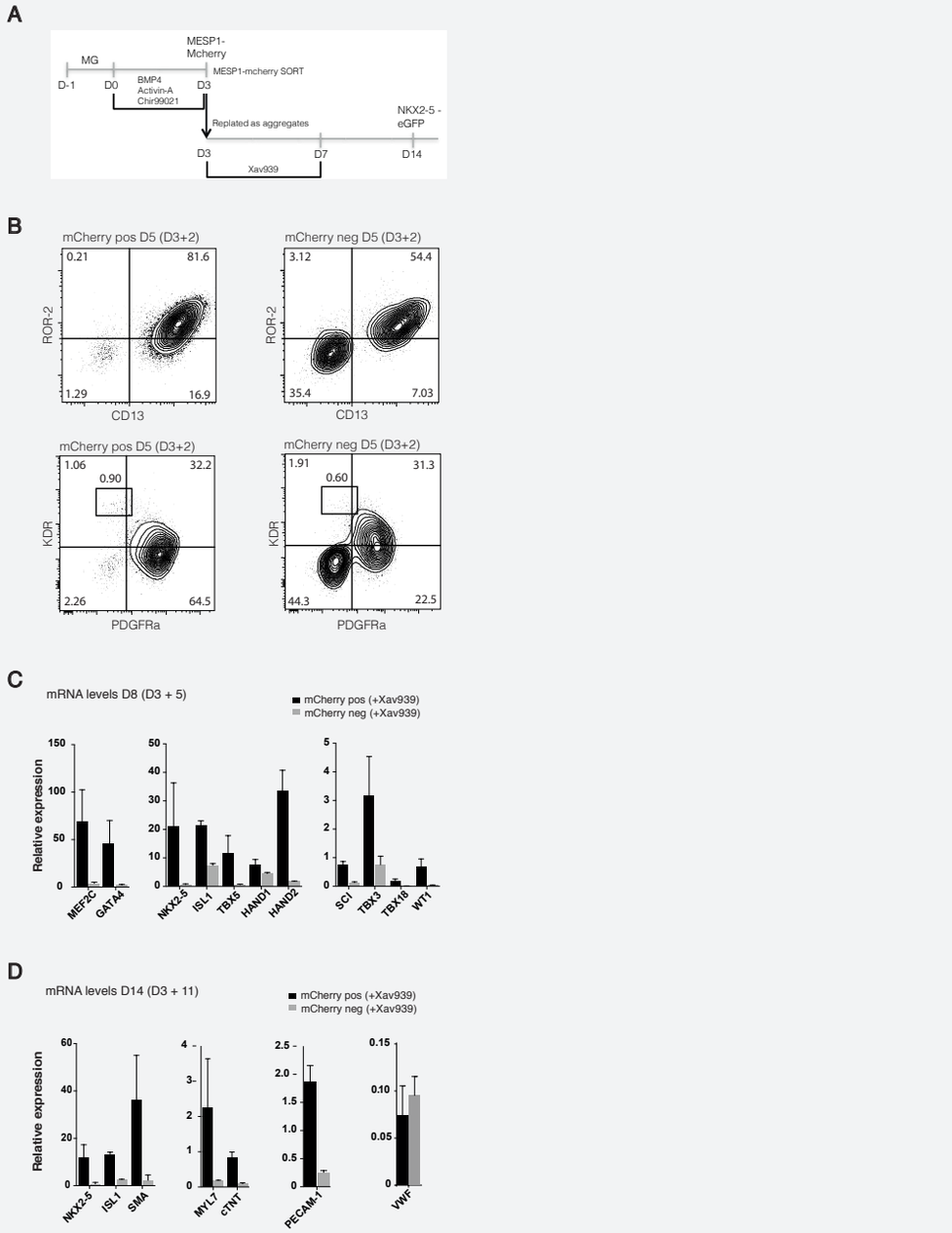

Figure 5. Cardiac differentiation induction from the MESP1-mCherry expressing pre-cardiac mesoderm progenitors. A: Schematic overview of the cardiac differentiation protocol for MESP1-mCherry cell fractions sorted by flow cytometry at day 3. **B:** Flow cytometric analysis of co-expression of cell surface proteins at differentiation day 5 (2 days after replating). **C,D:** Enrichment of mRNA transcripts of cardiac progenitor transcription factors at day 8 (n=3) and day 14 (n=2) in MESP1-mCherry pos and MESP1-mCherry neg derivatives (5 and 11 days after replating).

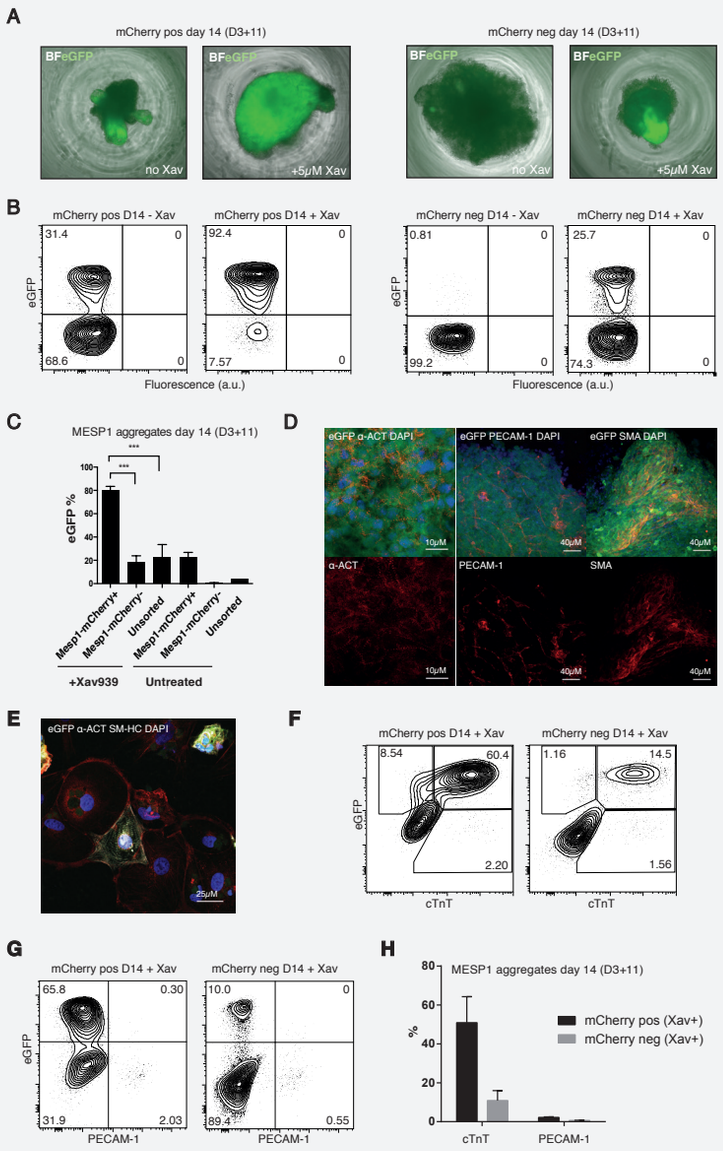

Figure 6. A: eGFP expression in MESP1-mCherry aggregates at day 14 of differentiation (11 days after replating), with and without treatment of Xav939. Brightfield (BF) overlay with eGFP. **B,C:** Percentage of cells expressing eGFP in day 14 MESP1-mCherry aggregates, with and without treatment of XAV939 (+sorted: n=7, -sorted: n=6, unsorted: n=2; error bars indicate SEM, ***p<0.001). **D:** Immunohistochemical stainings on day 14 MESP1-mCherry aggregates to analyse the presence of cardiac markers, including PECAM-1, SMA, SM-myosin, and α-actinin (α-ACT). **E:** Co-staining of α-ACT and SM-Heavy Chain (SM-HC) at d14 to discriminate smooth muscle cells from cardiomyocytes. **F,G,H:** Flow cytometric analysis of cTNT and PECAM-1 expression on day 14 MESP1-mCherry aggregates treated with XAV939 (n=2, error bars indicate SEM).

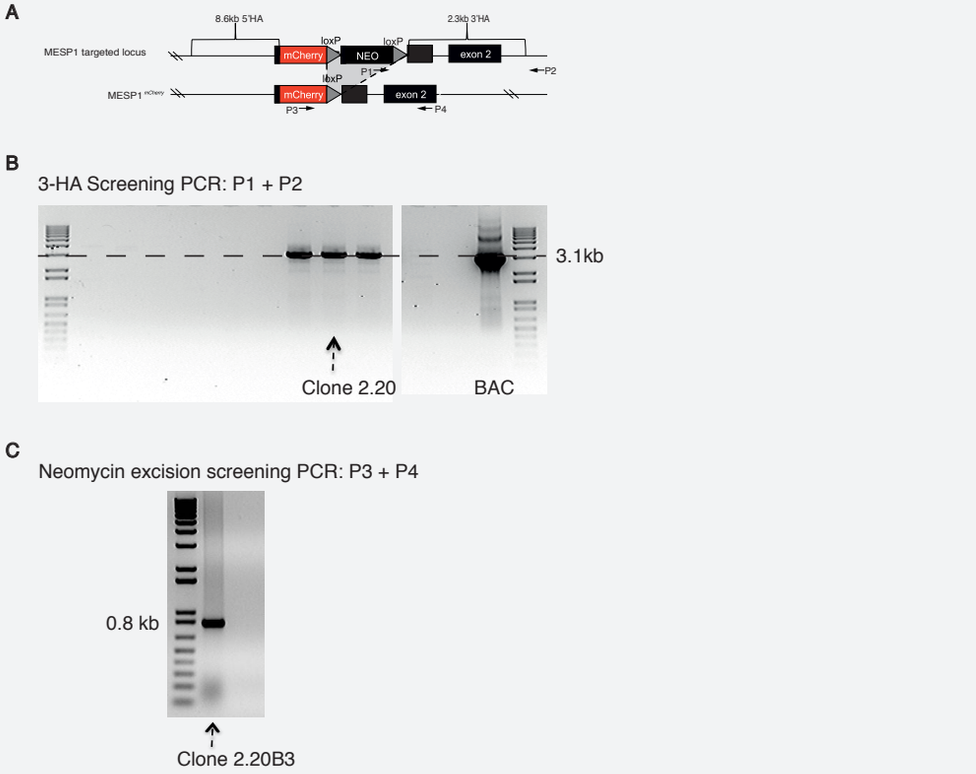

Figure S1. Genomic PCR experiments to confirm correct targeting of the MESP1 locus. A: Target screening PCR, using a forward primer annealing to the neomycin selection cassette and a reverse primer annealing outside the targeted region. Targeted clones show a PCR product of 3.1 kb. Clone 2.20 was used for neomycin cassette excision and further experiments. The modified Mesp1-mCherry bacterial artificial chromosome (BAC) was used as positive control. **B:** Neomycin excision of clone 2.20B3 was confirmed by a PCR reaction using a forward primer annealing within the mCherry cassette, and a reverse primer annealing within exon 2. Positively excised clones show a 800 bp PCR product.

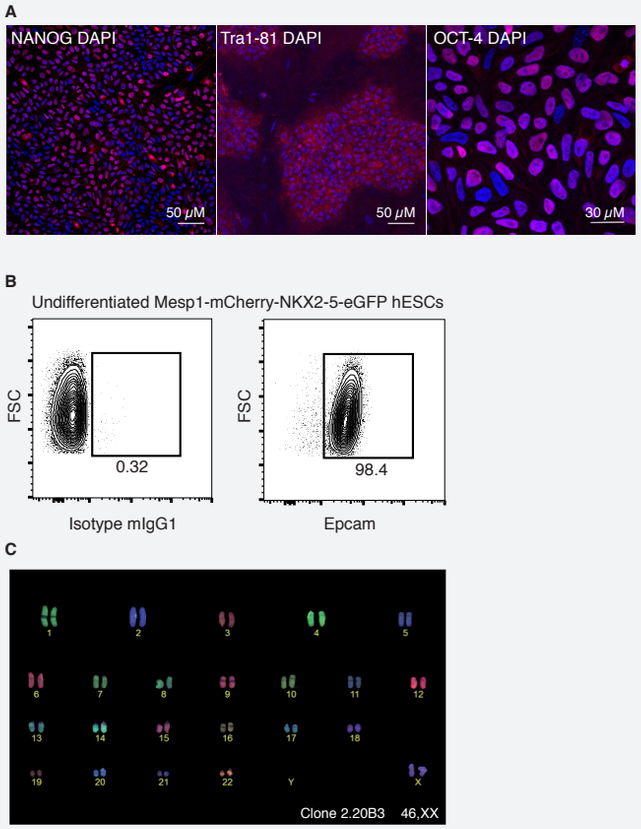

Figure S2. **A:** Immunohistochemical staining of MESP1^{mCherry}-NKX2-5^{eGFP} hESCs to confirm the expression of pluripotency markers after targeting. DAPI-stained hESCs (blue) with NANOG (red), OCT-4 (red), or TRA1-81 (red). **B:** Flow cytometric analysis of EPCAM expression on undifferentiated targeted hESCs. **C**: Karyotyping analysis of Clone 2.20B3 after targeting and neomycin cassette excision.

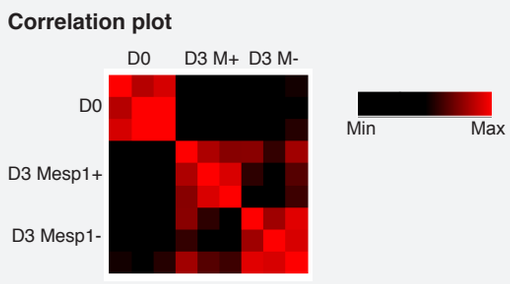

Figure S3. Additional information on MESP1-mCherry micro array experiments. **A:** Correlation heatmap between the three biological replicates of each timepoint: day 0 (D0), MESP1-mCherry positive cells (D3 M+), and MESP1-mCherry negative cells (D3 M-).

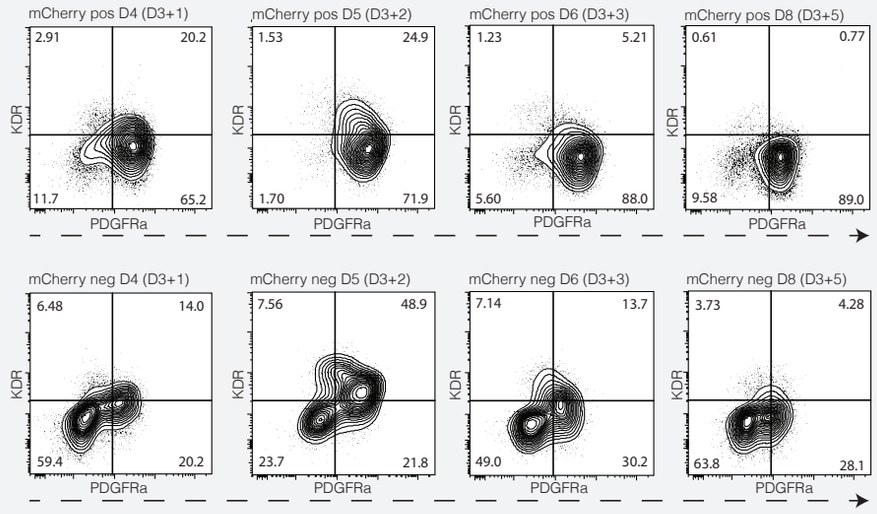

Figure S4. Expression profile of PDGFR-α and KDR during cardiac differentiation of MESP1-mCherry positive and negative sorted populations.

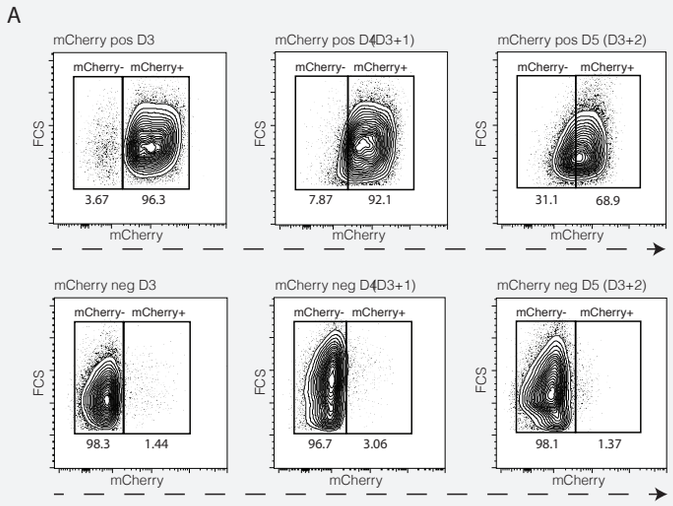

Figure S5. **A:** Re-analysis of mCherry expression on MESP1-mCherry pos and MESP1-mCherry neg sorted populations at day 3-5 of differentiation. **B:** mRNA expression levels of MESP1 and mCherry in MESP1-mCherry pos and MESP1-mCherry neg sorted populations at day 3 and 5 of differentiation.

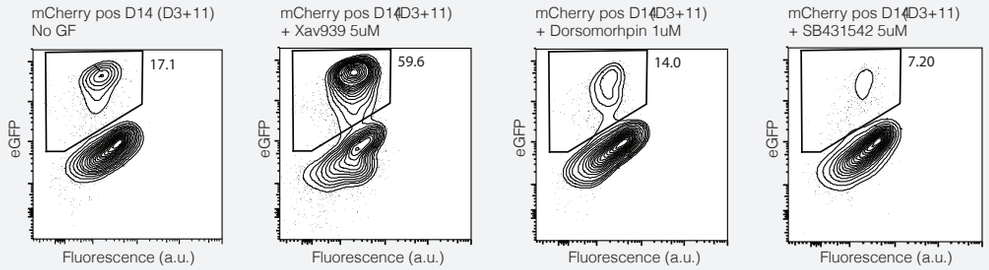

Figure S6. Effect of inhibition of specific signalling pathways after MESP1-mCherry sort and re-aggregation. NKX2-5-eGFP expression in MESP1-mCherry positive aggregates at day 14 of differentiation (11 days after replating). No GF: no addition of growth factors/inhibitors.

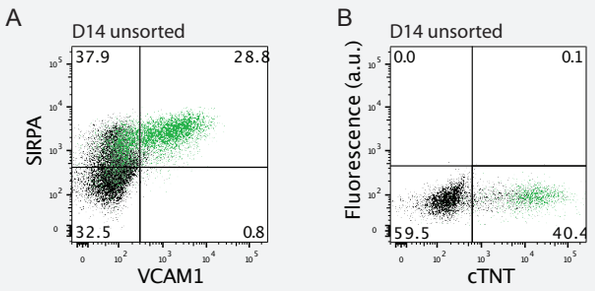

Figure S7. SIRPA+ VCAM1+ co-expression marks NKX2-5-eGFP+ cardiomyocytes. A: SIRPA+ VCAM1+ and **B:** intracellular cTnT expression on cardiac cells at day 14 of differentiation of NKX2.5$^{eGFP/w}$-hESCs. NKX2-5-eGFP+ cells are indicated in green.

Primer name	Primer sequence
MESP1_5arm_PvuI	5'GGAGAATTGCTTGAACCTGGGAGGTGGAGGCTGCAGTGAG CCGAGATCACGATCGCTCTCCTGAGTAGGACAAATC-3'
MESP1_3arm	3'CTTTAGTACATCCACAGAGTTGTGCAAGCATTGCCACTAATT TTAGAACGTTCCGCCTCAGAAGCCATAGAGC –5'
Target screen - F (P1)	5′- TCGCCTTCTTGACGAGTTCT -3′
Target screen - R (P2)	5′- AGGCAGCATGATGAGCCTTA -3′
Neo excision screen - F (P3)	5′- CCCCGTAATGCAGAAGAAGA -3′
Neo excision screen - R (P4)	5'-AGTTTCTCCCGCTCACTGG-3'

Supplementary table S1. Recombineering and screening primers.

Genes	Forward primer	Reverse primer
HARP	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
MESP1	CTCTGTTGGAGACCTGGATG	CCTGCTTGCCTCAAAGTG
MCHERRY	CAAGTTGGACATCACCTCCCAC	ACTTGTACAGCTCGTCCATGC
MESP2	TCTCCAGAGCCCTGTCTGTC	GTCCCTGGTCCTCTGAGTTG
E-CADHERIN	ATTCTGATTCTGCTGCTCTTG	AGTCCTGGTCCTCTTCTCC
SOX2	AACCCCAAGATGCACAACTC	GCTTAGCCTCGTCGATGAAC
BRACHYURY T	ATCACCAGCCACTGCTTC	GGGTTCCTCCATCATCTCTT
EOMES	TCTGTCCCATTGAGCTTCTC	TCTGTCCCATTGAGCTTCTC
MIXL1	GGTACCCCGACATCCACTT	GAGACTTGGCACGCCTGT
HAND1	AATCCTCTTCTCGACTGGGC	TGAACTCAAGAAGGCGGATG
GATA6	TCCCCCACAACACAACCTAC	TGTAGAGCCCATCTTGACCC
SNAI2	CAGACCCTGGTTGCTTCAA	TGACCTGTCTGCAAATGCTC
FOXC1	CCTGCTTGCCTCAAAGTG	TCCTTCTCCTCCTTGTCCTT
MEF2C	CCACTGGCTCACCCTTCTCTGC	ACCCGTTCCCTGCACTAGTG
NKX2-5	TTCCCGCCGCCCCCGCCTTCTAT	CGCTCCGCGTTGTCCGCCTCTGT
ISL1	TGATGAAGCAACTCCAGCAG	GGACTGGCTACCATGCTGTT
GATA4	GACAATCTGGTTAGGGGAAGC	GAGAGATGCAGTGTGCTCGT
TBX5	ACATGGAGCTGCACAGAATG	TGCTGAAAGGACTGTGGTTG
SCI	AAGGGCACAGCATCTGTAGTCA	AAGTCTTCAGCAGAGGGTCACGTA
HAND2	ACATCGCCTACCTCATGGAC	TGGTTTTCTTGTCGTTGCTG
TBX3	CGGTTCCACATTGTAAGAGC	TTCGGGGAACAAGTATGTCC
TBX18	TTGCTAAAGGCTTCCGAGAC	AGGTGGAGGAACTTGCATTG
WT1	TGTGTGCTTACCCAGGCTGC	TGTGTGCTTACCCAGGCTGC
IRX3	GTGCCTTGGAAGTGGAGAAA	TAGGATGAGGAGAGAGCCGA
MYL4	TCAAAGAGGCCTTTTCATTG	CGTCTCAAAGTCCAGCATCT
CD45	ACTCTTGGCATTTGGCTTTG	CACTGGGCATCTTTGCTGTA
MYOGENIN4	CAGCTCCCTCAACCAGGAG	GCTGTGAGAGCTGCATTCG

Supplementary table S2. Quantitative PCR primers for human genes.

GO Accession	GO Term Name	P-value
GO:0048856	anatomical structure development	7,13E-19
GO:0048731	system development	3,51E-18
GO:0009653	anatomical structure morphogenesis	5,91E-18
GO:0007275	multicellular organismal development	7,23E-18
GO:0044767	single-organism developmental process	1,50E-17
GO:0009887	organ morphogenesis	4,15E-16
GO:0001501	skeletal system development	5,47E-16
GO:0007369	gastrulation	7,80E-16
GO:0044763	single-organism cellular process	1,75E-15
GO:0044707	single-multicellular organism process	7,82E-15
GO:0001704	formation of primary germ layer	1,47E-14
GO:0048598	embryonic morphogenesis	3,99E-14
GO:0048513	organ development	2,39E-13
GO:0048729	tissue morphogenesis	2,42E-13
GO:0009790	embryo development	2,63E-13
GO:0030154	cell differentiation	3,81E-13
GO:0050793	regulation of developmental process	4,65E-13
GO:2000026	regulation of multicellular organismal development	9,09E-13
GO:0009888	tissue development	1,02E-12
GO:0007155	cell adhesion	1,25E-12
GO:0003002	regionalization	2,33E-12
GO:0048869	cellular developmental process	3,11E-12
GO:0048568	embryonic organ development	4,28E-12
GO:0005578	proteinaceous extracellular matrix	5,21E-12
GO:0009952	anterior/posterior pattern specification	6,27E-12
GO:0072358	cardiovascular system development	1,17E-11
GO:0072359	circulatory system development	1,17E-11
GO:0048468	cell development	1,51E-11
GO:0048585	negative regulation of response to stimulus	1,74E-11
GO:0022603	regulation of anatomical structure morphogenesis	2,07E-11
GO:0045595	regulation of cell differentiation	2,22E-11
GO:0007498	mesoderm development	3,07E-11
GO:0032989	cellular component morphogenesis	3,11E-11
GO:0048646	anatomical structure formation involved in morphogenesis	3,58E-11
GO:0007154	cell communication	3,88E-11

Supplementary table S3. Gene Ontology classes overrepresented in day 3 MESP1-mCherry pos cells vs. day 3 MESP1-mCherry neg cells (LogFC>+0.5, P<0.01).

Cell surface antigens
Vioblue-conjugated-mouse-anti–EPCAM (Miltenyi Biotec, 130-098-092)
Mouse-anti-SSEA4 (Santa Cruz, SC59368)
BV421-conjugated-mouse-anti-PDGFRα (BD Biosciences, 562799)
BV421-conjugated-mouse-anti-CD13 (Biolegend, 301716)
A488-conjugated-mouse-anti-ROR-2 (R&D, FAB2064G)
PE-conjugated-mouse-anti-FLK1/VEGFR2/KDR (R&D Systems, FAB357P)
APC-conjugated-mouse-anti-PECAM-1 (eBiosciences, 17-0319)
APC-conjugated-anti-SIRPA (Miltenyi Biotec)
PE-conjugated-anti-VCAM1 (BD Biosciences)
Pluripotency markers
Mouse-anti-TRA-181 (Millipore, MAB4381)
Mouse-anti-OCT3/4 (Santa Cruz, sc5279)
Anti-NANOG (Abcam, Ab21603)
Cardiac markers
Rabbit-anti-smooth muscle actin (Abcam, Ab5694)
Mouse-anti-cardiac Troponin T (CT3, Developmental Studies Hybridoma Bank, University of Iowa)
Mouse-anti-PECAM-1 (Dako, JC70A)
Rabbit-anti-α-actinin (Abcam, Ab11007)
Rabbit-anti-myosin smooth muscle heavy chain (BTI Biomedical Technologies, BT-562)

Supplementary table S4. Antibodies.

SUPPLEMENTAL METHODS

Analysis of MESP1-mCherry Derivatives

MESP1-mCherry-sorted cells were re-analyzed on mCherry expression by flow cytometry on day 3 (immediately after sorting), 4 and 5 of differentiation (1 and 2 days after reaggregation of days sorted cells, respectively). Analysis on KDR and PDGFR-α expression on day 4, 6, and 8 of differentiation of MESP1-mCherry-sorted cells was performed as described before.
To measure RNA levels of mCherry and MESP1 at day 3 and day 5 of differentiation (0 and 2 days after sorting), RNA isolation and qRT- PCR were performed as described before.

Inhibition of specific signalling pathways after MESP1-mCherry sorting and re-aggregation.

MESP1-mCherry cells were differentiated, sorted, and re-aggregated as described before. Pathway inhibitors assessed were 5uM XAV939, 5uM SB431542 (#1614, R&D), and 1uM dorsomorphin (P5499, Sigma Aldrich), from day 3 until day 7. NKX2-5-eGFP expression was analysed by flow cytometry at day 14 of differentiation (11 days after sorting), as described before.

NKX2-5eGFP/w hESC Culture and Cardiac Differentiation

NKX2-5$^{eGFP/w}$ hESCs were cultured in E8 medium (Life Technologies) according to the manufacturer's protocol. Cells were differentiated in BPEL as described previously, with FACS analysis performed at d14, using the conjugated antibodies SIRPA-APC (Miltenyi Biotec) and VCAM1-PE (BD Biosciences). Flow cytometric analysis of Troponin T was performed as described previously.

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