Guide to the heart: Differentiation of human pluripotent stem cells towards multiple cardiac subtypes

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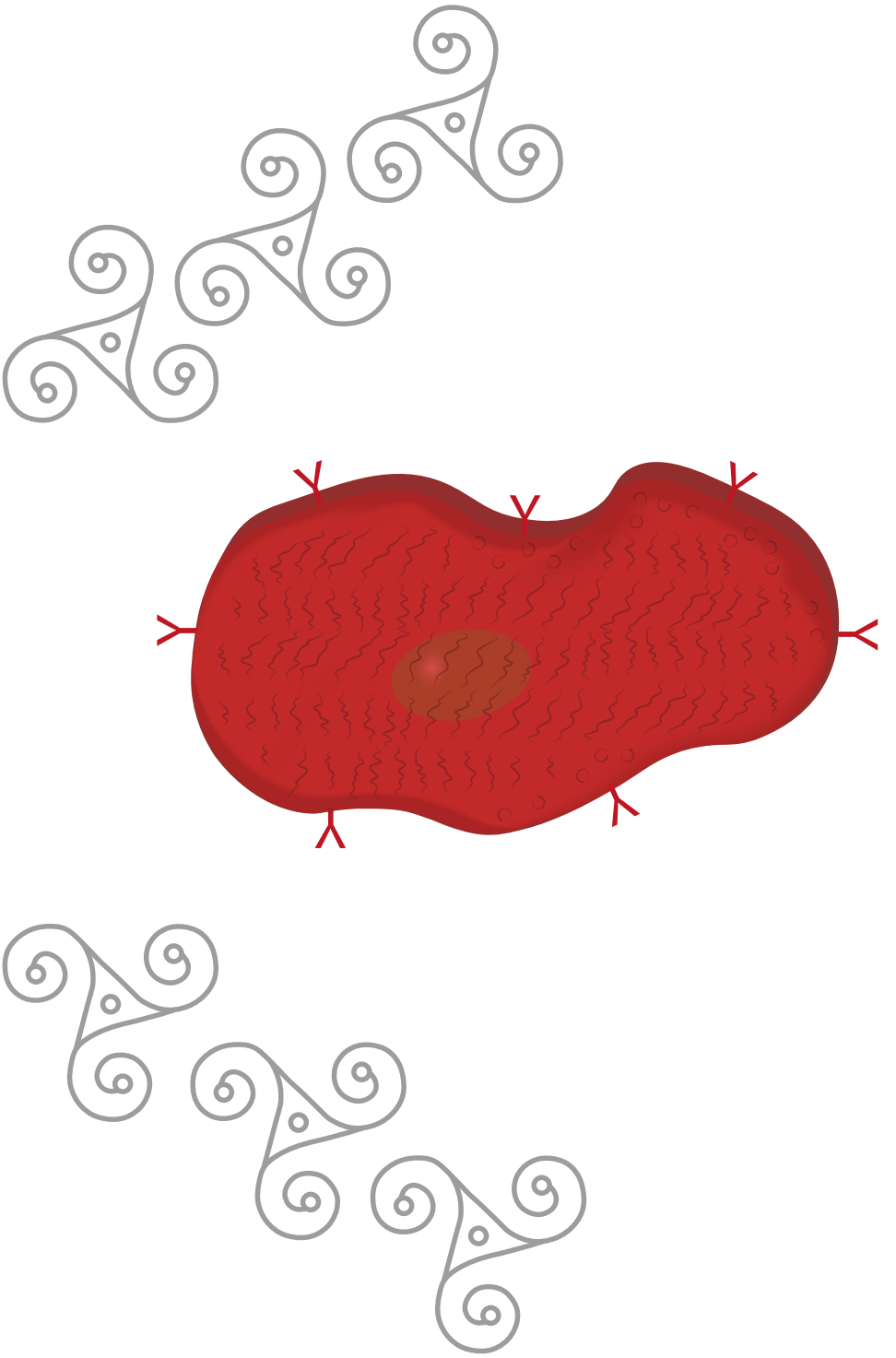

Chapter 4:

Cardiac subtype-specific surface markers for the selection of atrial and ventricular cardiomyocytes

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Abstract

Identification of atrial-specific surface markers is important for purification of atrial human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs), which may improve our knowledge on mechanisms underlying atrial specification, facilitate screening of atrial-selective drugs and modeling of atrial-specific disorders, such as atrial fibrillation. To identify human subtype-specific surface markers for the separation of atrial and ventricular CMs from mixed hPSC-derived cultures, we performed transcriptional profiling of hPSC-CMs by whole genome microarray analysis. Potential atrial and ventricular surface markers were validated by quantitative-PCR. We demonstrated enhanced transcriptional levels of selected candidate cell surface markers in atrial CMs, while selected ventricular cell surface markers were enriched in ventricular cells. In contrast, immunostaining and flow cytometric analysis of the same cell surface markers did not display significant differential expression pattern between cardiomyocyte subtypes. Strategies to induce a more adult phenotype may be important for the identification of subtype-specific surface markers.

Abbreviations:

CM – Cardiomyocyte; hESCs - Human embryonic stem cells; hESC-AM- Atrial-like cardiomyocyte; hESC-VM - Ventricular-like cardiomyocyte; RA - Retinoic acid

Introduction

Previous identification of cell surface markers, SirpA and VCAM1, for isolation of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) has been very helpful to facilitate studies on human cardiomyogenesis, cardiac disease, as well as pre-clinical pharmacology. Similarly, discovery of atrial-specific surface markers would allow purification of atrial CMs to better understand mechanisms of atrial specification, improve drug screening of atrial-selective treatments or model atrial-specific disorders, such as atrial fibrillation *in vitro*. Whereas most differentiation protocols yield a majority of ventricular CMs mixed with other cardiac subtypes (Blazeski et al., 2012), we recently showed efficient generation of human atrial CMs by modulating retinoic acid (RA) signaling during directed cardiac differentiation of human embryonic stem cells (hESCs) (Devalla et al., 2015).

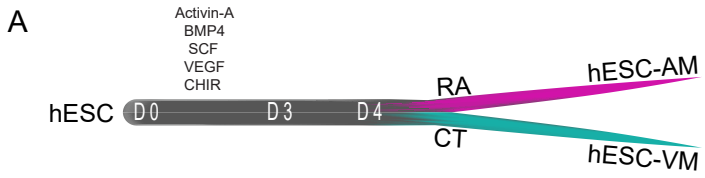
In murine embryonic and adult CMs, the spatiotemporal expression of surface integrin α6 (ITGA6) allows separation of atrial and ventricular subtypes from heterogeneous populations (Wiencierz et al., 2015). However, the expression of surface markers cannot be automatically extrapolated to the human situation (De Sousa Lopes et al., 2006).

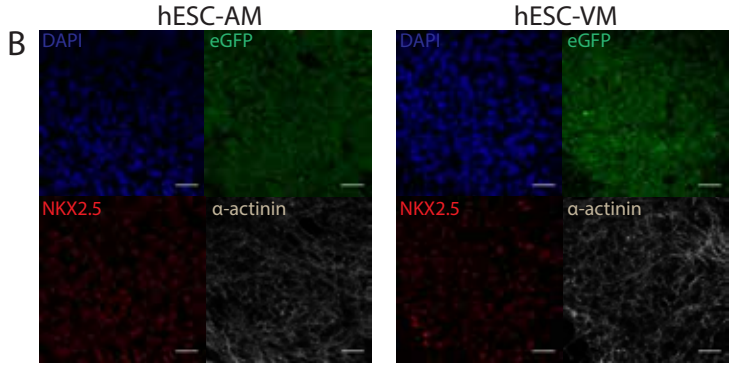
Here, we performed transcriptional analysis of hESC-CMs from directed atrial and ventricular differentiations by whole genome microarray to identify human subtype-specific surface markers that would allow the selection of atrial and ventricular CMs from mixed cultures. Upon identification, we validated potential targets by qPCR. Additionally, using specific antibodies we performed immunostaining and flow cytometric analysis. In contrast to the differential transcriptional expression levels, we did not observe significant differences for the same markers at the protein level between hPSC-AMs and hPSC-VMs subpopulations. The discrepancy between mRNA and protein expression may be a consequence of the immaturity of hPSC-CMs.

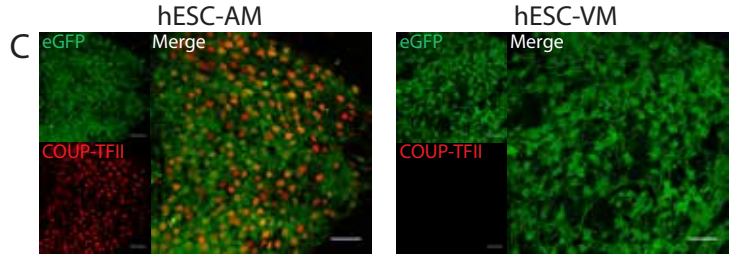
Results and Discussion

Transcriptional analysis to identify cardiac subtype-specific surface markers

For differentiation of hPSC to cardiomyocytes we used the NKX2.5$^{eGFP/+}$ fluorescent stem cell reporter line, which allows the visualization of GFP^{+} cardiomyocytes based on the activity of the early cardiac transcription factor NKX2.5, as described previously (Elliott et al 2011). To guide differentiation towards the atrial fate, 1 μM RA was added between day 4 and 7 (Devalla et al., 2015) (Figure 4.1A). As expected, both cardiac subtypes, RA-treated hESC- AMs and hESC-VMs from control condition (CT, no RA-treatment) co-expressed the cardiac markers NKX2.5 and α-actinin (Figure 4.1B), but only hESC-AMs displayed upregulation of atrial-enriched chick ovalbumin upstream promoter transcription factors II (COUP-TFII or NR2F2), thereby validating atrial identity of RA-treated CMs (Figure 4.1C). Human whole genome-wide transcriptional profiling by microarray hybridization of differentiated atrial and ventricular CMs after GFP-based purification at day 31. Using a 2-fold cut-off, 292 genes showed increased expression in hESC-AMs compared to hESC-VMs. In contrast, a total of 151 genes showed increased expression of more than twofold in hESC-VMs compared to hESC-AMs. We identified strong upregulation of potential cell surface markers for atrial CMs: *RAB9B* (Ras-related protein Rab-9B), *RASD1* (ras related dexamethasone induced 1), *CD83, FZD7* (Frizzled 7) and *GPR37* (G protein-coupled receptor 37) in hESC-AMs (Figure 4.1D). Similarly, microarray revealed enhanced expression of *TMEM71* (Transmembrane Protein 71), *SORCS1* (Sortilin-Related VPS10 Domain Containing Receptor 1) and *TSPAN32* (tetraspanin 32) in hESC-VMs (Figure 4.1E).







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Potential atrial surface markers

	hESC-AM	hESC-VM
RAB9B	2.40	1.00
CD83	2.70	1.00
FZD7	4.90	1.00
RASD1	2.54	1.00
GPR37	2.96	1.00

Potential ventricular surface markers

	hESC-AM	hESC-VM
TMEM71	1.00	3.45
TSPAN32	1.00	2.14
SORCS1	1.00	2.15

Figure 4.1: Potential surface markers of human stem cell-derived atrial (hESC-AM) and ventricular cardiomyocytes (hESC-VM). A) Atrial directed Spin-Embryoid body (Spin-EB) protocol and treatment with retinoic acid (RA). B) Immunostaining of cardiac NKX2.5 and α-actinin together with endogenous GFP expression and DAPI as nuclear staining in unsorted cultures after dissociation and re-plating. Scale bar = 100 µm. C) Immunostaining of COUP-TFII together with endogenous GFP expression in unsorted cultures after dissociation and re-plating. Scale bar = 100 µm. D and E) Genome wide transcriptional profiling by microarray. D) Heatmaps to display the fold difference of potential atrial and ventricular enriched surface markers in GFP-sorted RA-treated hESC-AM and CT hESC-VM at a threshold of 2-fold difference.

In agreement, based on GEO profiles or previous reports on their possible function in the heart, these potential candidate cell surface markers may play distinct roles in atria or ventricles. *RAB9B* is selectively expressed in human atrial, but not ventricular CMs *in vivo* (Lindskog et al., 2015). According to GEO profiles *FZD7* and *GPR37* are enhanced in atria when compared to ventricles. FZD7 is a WNT receptor, selectively expressed in murine atrial CMs from embryonic day 12.5 onwards. However, little is known about the function of these markers. RASD1 is highly expressed in rodent atrial CMs, but not ventricular CMs. Localized activation of RASD1 in the atria by different steroid hormones, like the glucocorticoids dexamethasone, corticosterone and estradiol, has been demonstrated to inhibit expression of the vasodilator atrial natriuretic factor (ANF) in a murine model of pressure overload (McGrath et al., 2012; Thapliyal et al., 2014). Not much is known about the expression or function of CD83 in the heart. Regarding selection of the ventricular cell surface markers, enrichment of transmembrane protein TMEM71 has been described in MESPI-cardiac derivatives together with the VCAM1, surface marker of hPSC-VMs (den Hartogh et al., 2016). In addition, the human protein atlas (www.proteinatlas.org) (Uhlén et al., 2015) describes a trend for increased expression in ventricles compared to atria. According to their GEO profile, *TSPAN32* and *SORCS1* are highly expressed in human ventricles, but can also be expressed in human right atrial appendage without history of atrial fibrillation at varying levels without defined explanation. For validation of the microarray data, we compared the expression of possible surface markers between sorted GFP-positive (GFP^+) hPSC-AM from RA and hPSC-VMs from CT, as well as GFP-negative (GFP^-) non-cardiac populations by quantitative-PCR (qPCR) (Figure 4.2). In agreement, we confirmed selective enrichment of potential surface markers in atrial and ventricular groups respectively (Figure 4.2A and B). *RAB9B, RASDI* and *GPR37* were exclusively expressed in hPSC-AMs, and not in any other population. Although highest expression of *CD83* and *FZD7* was observed in the RA^+ group, low expression was also detectable in GFP^- populations from RA and CT differentiations (Figure 4.2A).

Similarly, the potential ventricular marker *TMEM71* was selectively expressed in hPSC-VMs (CT^+). Expression of *TSPAN32* and *SORCS1* was also higher in the ventricular CT^+ group, although expression could also be detected in other populations as well (Figure 4.2B).

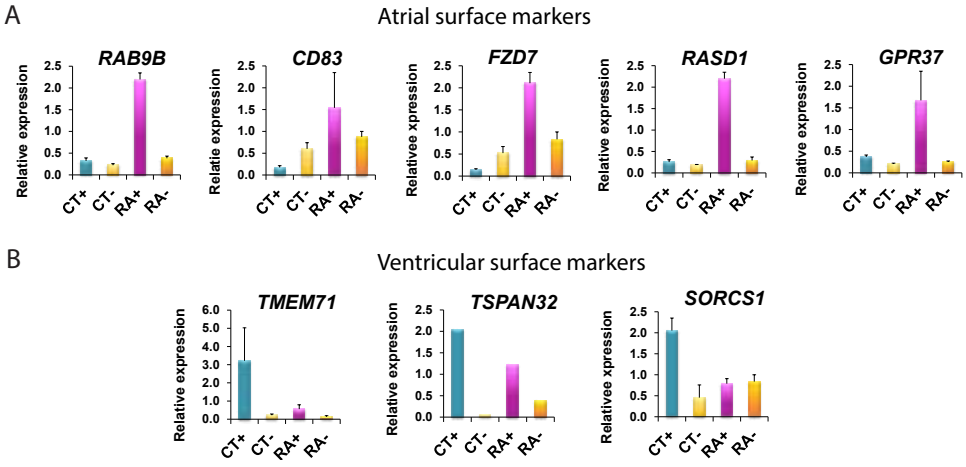

Figure 4.2: Expression levels in sorted CMs. Quantification by quantitative-PCR (qPCR) of potential atrial A) and ventricular B) enriched surface markers in GFP-sorted RA-treated hESC-AM (RA^+) and CT hESC-VM (CT^+) compared to GFP-negative (GFP^-) non-cardiac populations. Data are expressed as means ± SEM; (n=2).

To determine temporal expression changes, we then quantified expression levels of all potential surface markers at different time points between day 7 and day 28 of differentiation in mixed cultures with the cardiac marker cardiac Troponin (*TNNT2*) as monitor of cardiac differentiation (Figure 4.3). Expression of most potential atrial surface markers was rather similar in RA and CT condition until day 10 of differentiation and developed distinct expression patterns upon day 14. However, *RAB9B* was expressed at low levels in both populations until day 14 and only demonstrated distinct atrial expression from day 21 onwards (Figure 4.3B). Potential ventricular surface markers were differentially expressed as early as day 10 and maintained their enriched ventricular expression pattern until at least day 28 (Figure 4.3C).

In summary, transcriptional analysis revealed several potential cell-surface markers for cardiomyocyte subtype populations. Based on these results, *RAB9B* may only be used beyond day 21 of differentiation.

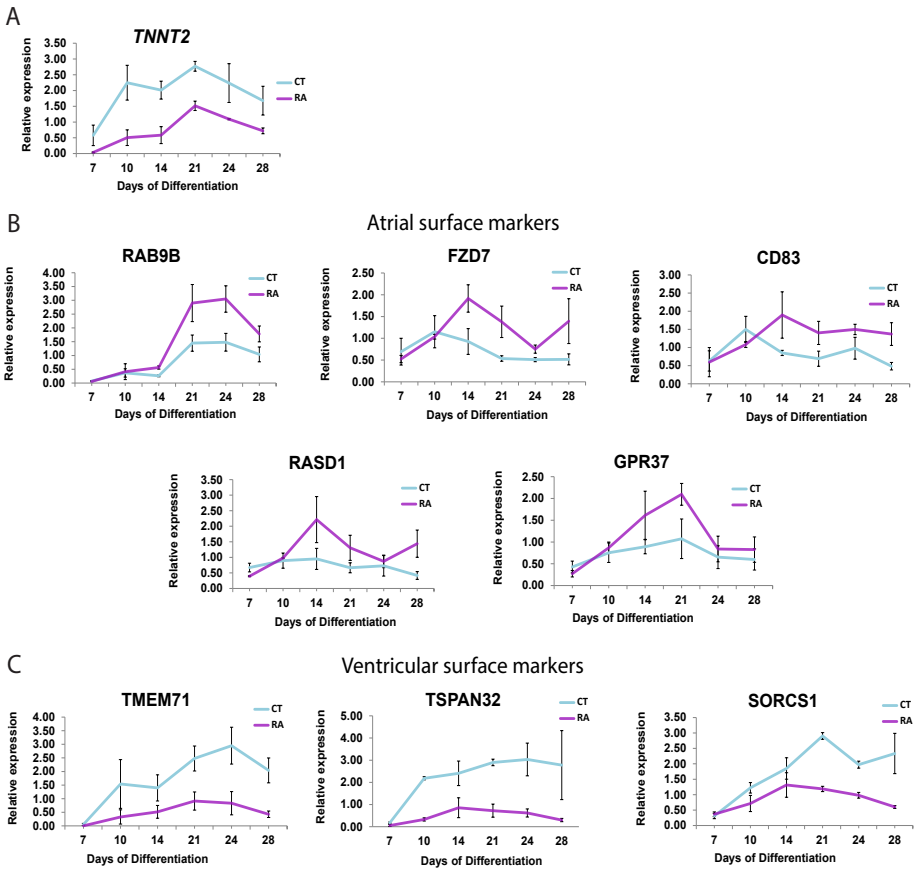

Figure 4.3: Temporal expression pattern of potential surface markers. A) Quantification of cardiac Troponin (*TNNT2*) in unsorted RA (purple) and CT (blue) cultures at several time points during differentiation. B) mRNA expression of potential atrial and C) ventricular B) enriched surface markers by quantitative-PCR (qPCR). Data are expressed as means ± SEM; (n=3).

In order to compare our results against human fetal atria and ventricles, we analyzed the transcriptional profile of right and left atria and corresponding ventricles by qPCR in one fetal heart with 11.2 weeks of gestation (Figure 4.4A and B). Besides *FZD7* all potential atrial surface markers were higher expressed in the left atrium than the right atrium. *FZD7* was the only marker that was predominantly expressed in the left atrium (Figure 4.4C). The other atrial markers were also expressed in left and even higher in right ventricles,

albeit at lower levels than in left atria for *CD83, RASD1* and *GPR37* (Figure 4.4C). Together these preliminary results from one fetal heart suggest that all selected atrial markers with the exception of *RAB9B* may be suitable for the selection of hPSC-AMs and primary fetal CMs of the left, but not of the right atrium. R*AB9B* may be useful for the separation of left and right atrial CMs, as well as left and right ventricular CMs. Since *FZD7* was only expressed at very low levels in ventricle, it may represent a suitable marker for selection of atrial CMs. However, analysis was performed in only one fetal heart. In addition, atrial and ventricular samples were not composed of pure CMs, but also included other cells types of the heart, such as endothelial cells, smooth muscle cells or fibroblasts. Increased expression of potential surface markers in one of these cell types may distort results. Interestingly, the direct comparison of the transcriptional data from *in vitro* samples and primary human fetal samples suggested that hPSC-AMs generated by modulating RA might more closely resemble CMs from the left fetal atrium. In agreement, hPSC-AMs are characterized by enhanced expression of the transcription factor PITX2 (Devalla et al., 2015), which is upregulated in left atrium (Kirchhof et al., 2011). However, this needs to be confirmed in follow-up studies.

The potential ventricular surface marker *TMEM71* was highly expressed in the right ventricle, but to a lesser extent also in the left atrium (Figure 4.4D). Importantly, *TSPAN32* was exclusively expressed in both left and right ventricles (Figure 4.4D). In contrast, *SORCS1* demonstrated enhanced expression in the right ventricle and also in the left atrium (Figure 4.4D). Preliminary analysis of the transcriptional expression in one fetal heart sample implies that *TSPAN32* is most suitable for the isolation of right and left ventricular CMs, while *TMEM71* and *SORCS1* on the other hand could be valuable for the separation of CMs from right and left ventricles.

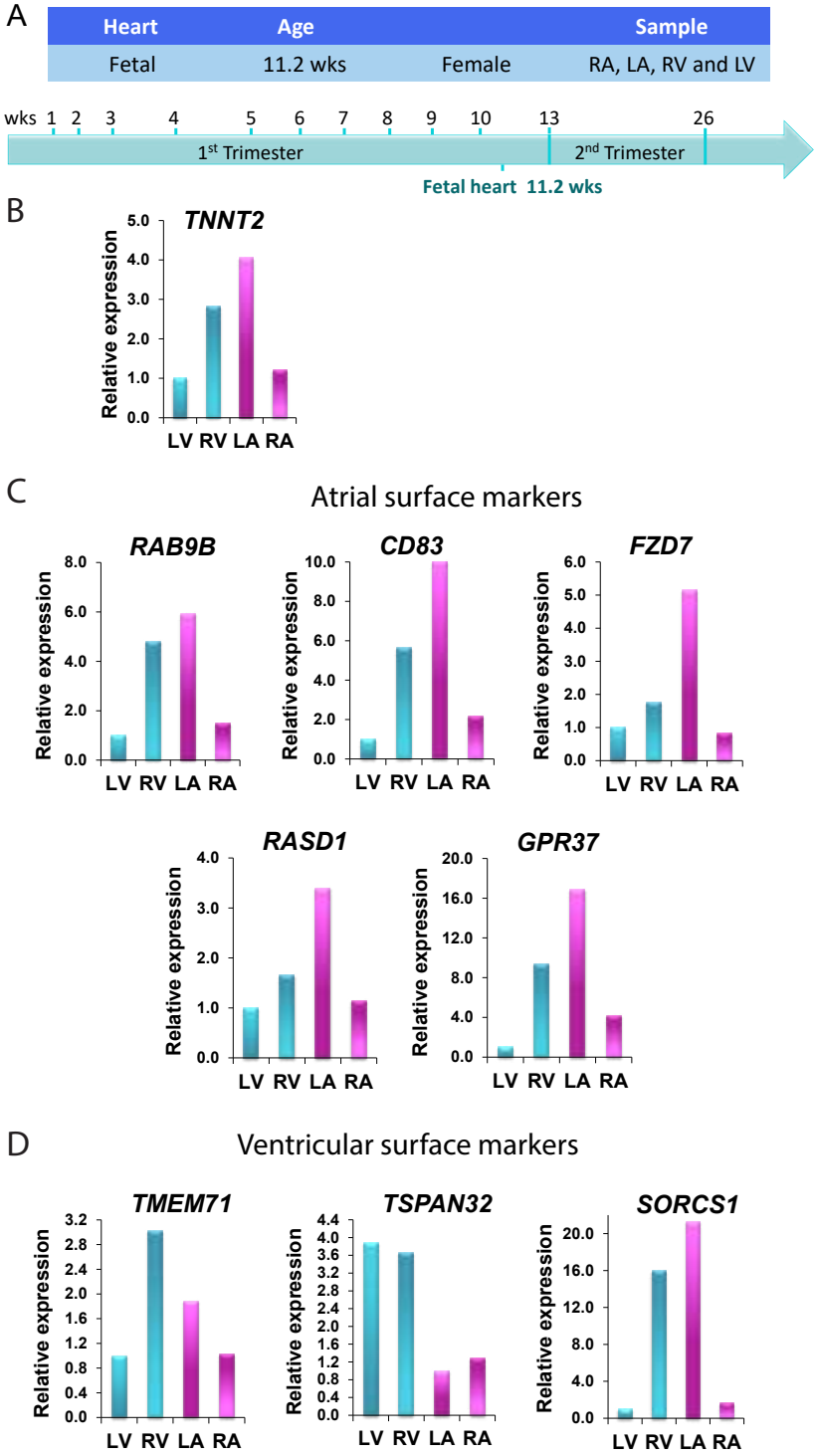
A
Heart
Age
Sample
Fetal
11.2 wks
Female
RA, LA, RV and LV
wks 1 2 3 4 5 6 7 8 9 10 13 26
1st Trimester
2nd Trimester
Fetal heart 11.2 wks
B
TNNT2
Relative expression
LV RV LA RA
C
Atrial surface markers
RAB9B
CD83
FZD7
RASD1
GPR37
D
Ventricular surface markers
TMEM71
TSPAN32
SORCS1

Figure 4.4: Expression of potential surface markers in human fetal heart. A) Samples of the right (RA) and left atrium (LA), as well as right (RV) and left ventricle (LV) of first trimester human fetal heart. B) Quantification of cardiac Troponin (*TNNT2*). C) mRNA expression of potential atrial and D) ventricular enriched surface markers by quantitative-PCR (qPCR); (n=1).

Validation of potential surface markers

The next step was to validate selected surface markers by immunostaining and flow cytometry. Since FZD7 and CD83 appeared to be most optimal for selection of atrial CMs, both targets were tested first. Fluorescent immunostaining of dissociated cells at day 21 of differentiation demonstrated prominent presence of FZD7 and CD83 in atrial and ventricular cultures (Figure 4.5B). In contrast, flow cytometry of samples did not reveal enrichment of both surface markers in hPSC-AMs (Figure 4.5B). Despite its non-selective expression in fetal heart samples, RAB9B was highly expressed in hPSC-AMs, but not in the other cell population. Thus, we analyzed RAB9B expression by immunostaining of dissociated cells at day 21 of differentiation (Figure 4.5C). Similar to immunostainings of CD83 and FZD7, we found expression of RAB9B in both cultures (Figure 4.5C). Based on human *in vivo* data, RAB9B is expressed in intercalated discs of atrial, but not ventricular CMs (Lindskog et al., 2015). Intercalated discs form the connection between adjacent CMs and are important for proper impulse propagation between individual cells. Since RAB9B was not localized to the intercalated discs our samples, this may suggest that the immature phenotype of both atrial and ventricular CMs complicate the identification of subtype-specific surface markers. Discrepancy between transcriptional expression and protein has previously been reported (van den Berg et al., 2017a, 2017b), technical advancements, such as single cell RNA-sequencing or protein-based array systems may lead to the identification of distinct surface markers.

To disclose if difficulties in identifying an atrial surface marker are related to the immature character of the CMs, we performed immunostainings for the gap junction connexins (Cx) 40 and 43. Fast gap junction protein Cx 40 is the primary Cx in fast depolarizing atrial CMs, which express only little Cx 43; in ventricles Cx 43 dominates and Cx 40 is almost absent (Davis et al., 1995). Cx expression in fetal CMs randomly localizes to the circumferential

membrane and only upon further development, Cx expression polarizes to the ends of the cells to form gap junctions between neighboring CMs. We identified prominent expression of both Cx 40 and 43 in hPSC-AMs and hPSC-VMs. This data implies that similar expression patterns of potential surface markers and also the gap junction proteins Cx 40 and 43 in hPSC-AMs and hPSC-VMs may reflect the immature phenotype of the cells. These findings suggest that culture conditions need to be further optimized which includes activation of specific signaling pathways, use of substrates mimicking native extracellular matrix conditions of the heart and 3D modeling, to further improve specification and maturation of cardiac subtypes and generate defined CMs of right and left atria and ventricles. However, it is also possible that technical challenges related to antibody specificity or staining conditions affected the outcome of our results.

Alternatively, the generation of lineage-specific reporter lines may enable selection of subtype CMs. Pure populations of CMs would support the identification of atrial and ventricular surface markers. Since, we have previously identified that the nuclear receptor transcription factor COUP-TFII is rapidly increased upon RA-treatment during differentiation to atrial CMs and not expressed in ventricular cardiomyocytes (Devalla et al., 2015), COUP-TFII may be an appropriate marker for generating an atrial reporter line in hPSC.

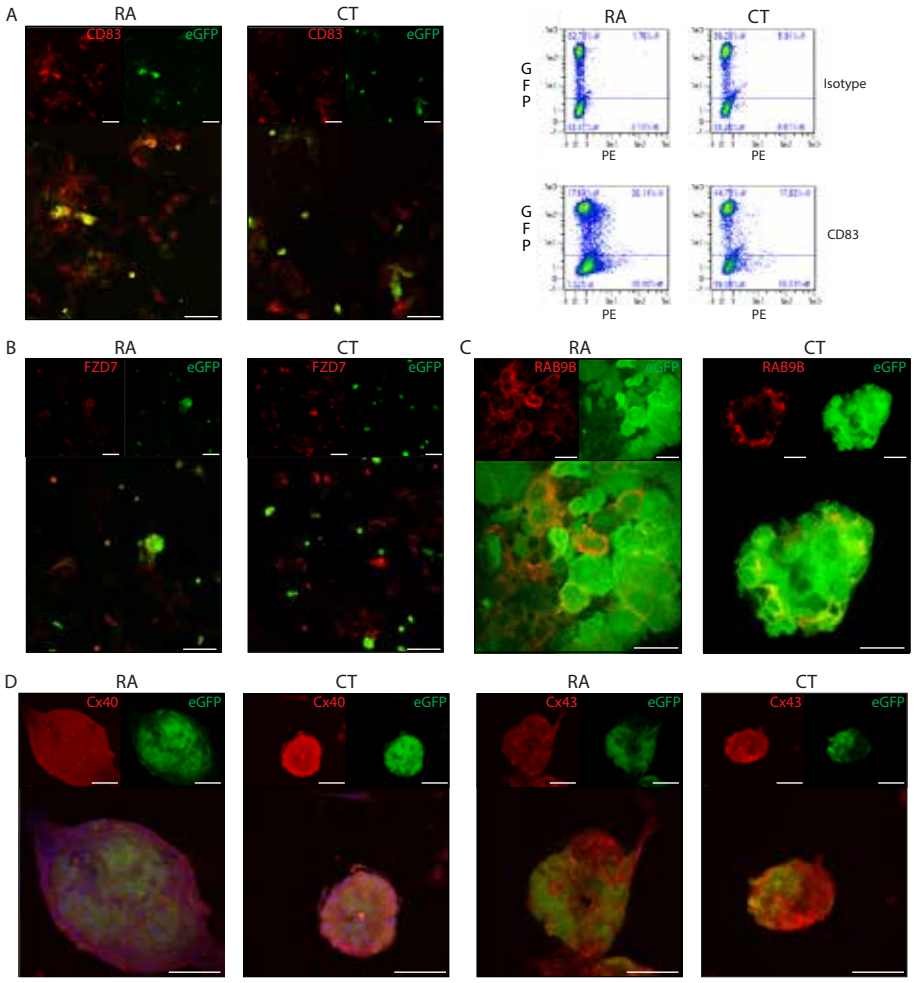

Figure 4.5: Validation of potential surface markers on protein expression level. A) Fluorescent immunostaining of the potential atrial surface marker CD83 together with endogenous GFP (eGFP) expression in unsorted RA and CT cultures after dissociation and re-plating. Scale bar = 100 µm. Representative flow cytometry plot of RA and CT samples after isotype or CD83 staining. B) Fluorescent immunostaining of the potential atrial surface marker FZD7 together with eGFP expression in unsorted RA and CT cultures after dissociation and re-plating. Scale bar = 100 µm. C) Fluorescent immunostaining of the potential atrial surface marker RAB9B together with eGFP expression. Scale bar = 100 µm. D) Immunostaining of the gap junction connexins (Cx) 40 (left panel) and 43 (right panel) together with eGFP expression in unsorted RA and CT cultures after dissociation and re-plating. Scale bar = 100 µm.

Materials and Methods

Maintenance of hESCs

HESCs were maintained as undifferentiated colonies on irradiated mouse embryonic fibroblasts (MEFs) in hESC medium containing DMEM-F12 (GIBCO, Thermo Fisher Scientific), non-essential amino acids (GIBCO, Thermo Fisher Scientific), ß-mercaptoethanol (GIBCO, Thermo Fisher Scientific), KnockOut Serum Replacement (GIBCO, Thermo Fisher Scientific) and basic Fibroblast Growth Factor (bFGF) (Miltenyi Biotech).

Cardiac differentiation of hESCs

Differentiation to hESC-derived CMs was performed using a previously described Spin-EB protocol (Devalla et al., 2015). Concisely, after harvesting, cells were resuspended in BPEL (Bovine Serum Albumin (BSA) Polyvinylalcohol Essential Lipids) medium supplemented with growth factors at a final concentration of 30 ng/ml BMP4 (R&D Systems), 30 ng/ml VEGF (Miltenyi Biotech), 40 ng/ml SCF (Miltenyi Biotech), 30 ng/ml Activin A (Miltenyi Biotech) and 1.5 µM CHIR 99021 (Axon Medchem). For atrial differentiations, 1 µM RA (Sigma Aldrich) was added from day 4 till 7 without additional refreshments. Following plating at day 7, BPEL was refreshed every 3-4 days.

Transcriptional analysis

Transcriptional analysis in sorted samples was performed at day 28 of differentiation and time course analysis of transcription at day 7, 10, 14, 21, 24 and 28. Total RNA was isolated with the NucleoSpin RNA isolation Kit (Macherey-Nagel) according to manufacturer's protocol and cDNA was synthesized with iScript cDNA Synthesis Kit (BIO-RAD). To analyze gene expression, microarrays were performed on a Human HT -12 v4 Expression Bead Chip at ServiceXS. Gene expression analysis was performed with GeneSpring GX (Agilent Technologies). Quantitative PCR (qPCR) was carried out in triplicate reactions for each target using SybrGreen master mix

(Applied Biosystems) and the CFX384 Real-time PCR detection system. Data was normalized to hARP as housekeeping gene.

Immunostaining and Confocal Imaging

D14 differentiations were dissociated and re-plated in TID medium (Birket et al., 2015b) on matrigel-coated glass coverslips. 1-2 weeks later, cells were fixed in 2% paraformaldehyde and were stained using primary antibodies to CD83 (Mouse anti-human CD83-PE; clone HB15e; BD Biosciences), FZD7 (Rat anti-human FZD7-APC; clone 151143; R&D systems) and RAB9B (Rabbit anti-human RAB9B; clone 18152-1-AP; Proteintech) or Connexin 40 and Connexin 43 followed by secondary conjugated antibodies to Alexa Fluor 647, 555 or Cy-3. Nuclei were stained with DAPI. Confocal images were acquired and analyzed using Leica Microsystems.

Flow cytometry

D21 RA and CT EBs were dissociated with 10x TrypLe Select and cells were stained for FZD7 or CD38 at RT for 20 min or at 4°C for 1 h and analyzed by flow cytometry. To eliminate cell debris or aggregated cells, events with very low or high side and forward scatter were excluded. Subsequent data analysis was performed with MACSQuantify™ Software.

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