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HUMAN C-KIT+CD45- CARDIAC STEM CELLS ARE HETEROGENEOUS AND DISPLAY BOTH CARDIAC AND ENDOTHELIAL COMMITMENT BY SINGLE-CELL QPCR ANALYSIS

Short title: Single-cell analysis of cardiac C-kit+CD45- cells

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Abstract

C-kit expressing cardiac stem cells have been described as multipotent. We have previously identified human cardiac C-kit+CD45- cells, but only found evidence of endothelial commitment. A small cardiac committed subpopulation within the C-kit+CD45- population might however be present. To investigate this at single-cell level, right and left atrial biopsies were dissociated and analyzed by FACS. Only right atrial biopsies contained a clearly distinguishable C-kit+CD45- population, which was single-cell sorted for qPCR. A minor portion of the sorted cells (1.1%) expressed early cardiac gene *NKX2.5* while most of the cells (81%) expressed late endothelial gene *VWF*. *VWF*- cells were analyzed for a wider panel of genes. One group of these cells expressed endothelial genes (*FLK-1, CD31*) while another group expressed late cardiac genes (*TNNT2, ACTC1*). In conclusion, human C-kit+CD45- cells were predominantly localized to the right atrium. While most of these cells expressed endothelial genes, a minor portion expressed cardiac genes.

Key words

FACS; cardiac stem cells; Single-cell qPCR; human; heart; atrium

Abbreviations

qPCR	Quantitative Real-Time Polymerase Chain Reaction
SP	Side Population
SEM	Standard Error of the Mean

Introduction

Traditionally, the heart has been regarded as a non-regenerative organ after the neonatal period. During the past ten years, this notion has been challenged. A slow turnover of cardiomyocytes have been shown by studying ^{14}C content in human cardiomyocytes [1]. C-kit+ stem cells residing in the myocardium have been suggested as the source of cardiac regeneration. These cells have been described as multipotent with the capacity to differentiate into cardiac, endothelial and smooth muscle cells [2]. Most studies however have been conducted in animal models which may not reflect the human situation. We have previously identified a population of C-kit+CD45- cells in human right atrial tissue [3]. These cells showed an endothelial profile both on gene and protein levels, and could not be induced to differentiate into cardiomyocytes. This is in line with a study in mice, which showed that the capacity of cardiac C-kit+ cells to differentiate into cardiomyocytes existed in the neonatal heart but was lost in the adult [4]. On the other hand, it has been suggested that C-kit+ cells in the adult heart are heterogeneous in regard of differentiation potential rather than truly multipotent. When C-kit+ cells were divided based on FLK-1 expression, the FLK-1+ cells showed endothelial differential potential whereas the FLK-1- cells could be induced to cardiac differentiation both *in vitro* and *in vivo [5,6]*.

From human cardiac tissue, biopsy material is rather limited and the C-kit+ population represents only a small fraction of the cells in a biopsy. Many studies have thus adopted a primary culture step before isolation of C-kit+ cells [3,7], which may induce expansion of one committed subpopulation relative to another. Furthermore, most studies have been conducted on right atrial tissue. However, it could be hypothesized that higher pressure on the left side of the heart could affect distribution and phenotype of stem cells.

In the present study, we wanted to compare distribution of C-kit+CD45- cells between right and left atrium and explore whether the C-kit+CD45- population in human adult heart show evidence of lineage commitment into the cardiac and endothelial lineages before expansion *in vitro*. For this purpose, we employed a novel single-cell based strategy where single C-kit+CD45- cells were sorted and subjected to quantitative PCR (qPCR) analysis of lineage and stem cell markers.

Material and methods

Tissue procurement

Atrial biopsies (n = 13) were obtained from patients undergoing Maze surgery at Sahlgrenska University Hospital, after informed written consent. The procurement of biopsies was approved by the local ethics committee at the University of Gothenburg and carried out in accordance with the Helsinki Declaration of 1975, as revised 2000. Biopsies from both left (n = 6, weight 1.4 - 5.0 g) and right (n = 7, weight 0.70 - 1.5 g) atrium were obtained. Age range of the included patients was 40 - 76 years. Of these right atrial biopsies, 5 was used for single cell sorting whereas two of the biopsies due to temporary problems with the sorting unit could only be used for data acquisition. Parts of the biopsy material from some of the patients were also used in other previously published studies [3,8] or unpublished studies due to the limited supply of biopsy matieral.

Cell isolation procedure

Biopsies were mechanically and enzymatically digested followed by en epitope regeneration step as described previously [3,8]. A detailed description of the cell isolation procedure is also available in the online supplement.

Flow cytometry and cell sorting

Cells were stained with 7-AAD (Invitrogen) for dead cell discrimination and antibodies (mouse anti C-kit-APC and mouse anti CD45-PE-Cy7, BD) for 30 minutes, then poured through a 40 µm cell strainer (BD) and washed twice with FACS staining buffer (PBS supplemented with 5% FBS, 1% BSA and 2 mM EDTA). FACS analysis was carried out on a FACSaria II cell sorter (BD). Data analysis was done using FACSdiva version 6.1.1 (BD). Background staining was determined by appropriate isotypic controls. For C-kit, a gating strategy to minimize the risk of false positive cells was used (isotypic control in the range of 0.002 - 0.02%). Gates for CD45 were on the other hand set to get as good discrimination between the C-kit+CD45- and C-kit+CD45+ populations as possible. Isotypic controls were subtracted when statistics were calculated. For qPCR, cells were sorted in single-cell sorting mode into 96-well plates with lysis buffer. Plates were cooled during sorting.

Single-cell qPCR analysis and cluster analysis

Lysis, cDNA-synthesis, preamplification and gene expression analysis was performed using the TaqMan® PreAmp Cells-to-C_T™ Kit (Life Technologies, Carlsbad, CA USA). Analysis was carried out according to manufacturer's description with minor modifications (see online supplement for detailed description). Analysis was carried out with a ABI7900HT instrument (Life Technologies). Since it is not possible to use a reference gene for relative quantification of gene expression on single-cell level [9], data was expressed as raw Ct values / cell. A positive control sample was included in all analyses carried out. This showed minimal variation between analyses. Cluster analysis of the *VWF*- or *NKX2.5*+ fraction of the *C-KIT*+ cells was carried out using GenEx v.5 software (MultiD Analyses AB, Gothenburg, Sweden). Ward's algorithm with eucledian distance was used both for gene and cell clustering.

Statistical analysis

FACS data is presented as mean ± standard error of the mean (SEM) of performed experiments. Numbers (n) of analyzed biopsies are stated in the figures. Statistical significance was determined using two-sided Student's t-test, group wise comparison. A value of $p<0.05$ was considered statistically significant. Statistical calculations were carried out using SPSS v.20 (IBM, New York, NY, USA) and Excel v.2003 (Microsoft, Redmond, WA, USA).

Results

Cardiac cells from right and left atrium were stained for C-kit and hematopoietic marker CD45. From right atrium, a small population of C-kit+CD45- cells could reproducibly be obtained (Fig. 1). In left atrial biopsies the C-kit+CD45- population was barely detectable and could not be sorted for single-cell analysis.

C-kit+CD45- cells from right atrial biopsies were sorted as single-cells (471 cells, 5 donors). True C-kit+CD45- identity was confirmed by gene expression analysis. About 75% of the sorted cells expressed *CKIT* while no cells expressed *CD45*. These cells were then analyzed for endothelial markers (*FLK-1*, *VWF*) and cardiac transcription factor *NKX2.5*. Most cells expressed endothelial markers, but there was also a small percentage of *NKX2.5*+ cells (Table 1).

We then wanted to study the non-endothelial *CKIT*+ population more closely. Since expression of *VWF* is indicative of late endothelial development, those cells were excluded from further analysis. Notably, most of the *VWF*+ cells co-expressed *FLK-1* (data not shown). *VWF*- cells were analyzed for genes of cardiac (*TNNT2, ACTC1, GATA4, MEF2C*), endothelial (*CD31*), smooth muscle (*ACTA2*), fibroblast (*DDR2*) and stem cell (*OCT4*) lineages. Since *NKX2.5*+ cells were considered to be of special interest, these were included regardless of *VWF* expression status. By 2-dimension cluster analysis, four groups of cells were identified (Fig. 2, Group A-D). Group A expressed cardiac genes but no endothelial markers. Group B consisted of two subpopulations where one expressed *FLK-1* and the other was positive for *ACTC1*. Group C was negative for all of the analyzed genes and Group D showed an endothelial profile with some cells expressing stem cell associated genes as well as a few cells expressing one or two cardiac genes. When looking at the gene cluster analysis (Fig. 2, X-axis), endothelial and cardiac genes respectively clustered together.

Discussion

C-kit+ cardiac cells have been described as clonogenic, multipotent stem cells with capacity to differentiate into endothelial, cardiac and smooth muscle cells. This has been shown both in animal models [2] and in humans [10]. However, other studies of adult C-kit+ cells have failed to identify cardiomyocyte differentiation or early commitment [3,4]. Furthermore, it has been shown that the C-kit+ population could be subdivided based on VEGF receptor FLK-1 expression, and that the cardiomyogenic potential was confined to the FLK-1- population [5,6]. One reason could be that the C-kit+ population is not homogenous but contains both cardiac and endothelial committed cells.

Few previous studies have investigated the distribution of cardiac stem cells. In mouse cardiac tissue, C-kit+ cells were predominantly confined to the atrium and apex. However, distribution in left and right atrium was not separately investigated [11]. In human cardiac tissue, expression of C-kit was determined in monolayer cultured cells from different localizations of the heart. Expanded cells from right atrium contained a higher percentage of C-kit+ cells compared to left atrium, which complies with our results [7]. Notably, the percentages of C-kit+ cells after expansion were about 1000-fold higher than what we detected in directly isolated cells. This underlines the profound alteration in cellular composition induced by *in vitro* expansion. The reason for difference in expression of C-kit+CD45- cells between right and left atrium could only be speculated about. The heart may contain other stem cell populations than the C-kit+ population, such as Side Population (SP) cells [12]. These cells, when isolated from the murine heart, have similarly to C-kit+ cells been able to differentiate into both cardiomyocytes [12] and endothelial cells [13]. In these studies, SP cells were found to be negative for C-kit expression. We have recently showed that human cardiac SP cells could only be detected in left atrial tissue [8]. It could thus be hypothesized that in left atrium, SP cells rather than C-kit+ cells contribute to tissue regeneration.

To investigate whether the human C-kit+CD45- population shows signs of lineage commitment *in vivo*, we used single-cell qPCR to measure gene expression in directly isolated cells. This technique has previously successfully been used to detect sub-populations among colon tumour cells [14]. Most of the C-kit+CD45- cells showed evidence of endothelial commitment based on expression of late endothelial marker *VWF*. These cells were mostly also *FLK-1*+ and it is reasonable to assume

that they represent the previously described C-kit+FLK-1+ endothelial progenitor population [5,6]. About 19% of the isolated *CKIT*+ cells were negative for *VWF*, suggesting heterogeneity within the C-kit+CD45- population. When these cells were analyzed for additional genes, cluster analysis revealed four groups of cells. The cardiac group showed expression of genes associated with late cardiac development, rather than early cardiac transcription factor genes. This is in contrast to previous studies by immunohistochemsitry, where a population of human cardiac C-kit+ cells expressing cardiac transcription factors either alone or in combination with cardiac structural proteins have been described [15,16]. This discrepancy could potentially be explained by that late cardiac genes are more abundantly expressed than transcription factors. Although sensitive, single-cell qPCR technique may fail to detect genes with low copy number. In our study, only a few *NKX2.5*+ cells were observed. All of them co-expressed at least one late cardiac gene. Paradoxical, most of theses cells also expressed late endothelial marker *VWF*. It has however previously been shown that a minor subpopulation of cardiomyocytes in the developing heart also co-expressed *VWF* [17]. Importantly, as the population of cardiac committed cells represented only a small fraction of all *CKIT*+ cells, expression of cardiac genes may be very low or undetectable if analyzing a larger group of C-kit+CD45- cells [3]. Moreover, the cardiac sub-population may be lost during *in vitro* expansion [4]. It should be noted that several previous studies, in contrast to our, have found a predominant cardiomyogenic commitment rather than endothelial of the C-kit+ population both in the murine [2] and human heart [6,16]. On the other hand, our results comply with the results of Jesty et al., showing that C-kit+ cells derived from adult mouse heart adopt an endothelial fate rather than a cardiomyogenic both in vivo and in vitro [18].

The endothelial group could be hypothesized as a more undifferentiated endothelial progenitor population compared to the *VWF*+ cells. Within this population a few cells were positive for cardiac genes. Those might represent cells with bi-potent differentiation capabilities. To our knowledge, there is no previous in vivo data available on such bi-potent C-kit+ progenitor population although it has been shown that C-kit+ FLK-1+ progenitors to a lesser degree can be induced to differentiate into the cardiomyogenic lineage *in vitro* [6].

One group of *CKIT*+ *VWF*- cells in the present study was negative for both endothelial and cardiac genes. Since they might be committed to other cell lineages found in the heart, a broader panel

of fibroblast, smooth muscle and stem cell genes were investigated but no expression was found. In previous studies of the normal mouse heart, a part of the C-kit+ cells displayed no lineage commitment by immunohistochemistry [2,19]. It could be hypothesized that the lineage negative group of cells identified in our study corresponds to this previously described subpopulation. The lack of expression of stem cell associated *OCT4* in this group is unexpected in this regard but have previously been noted in cardiac derived Sca-1+ progenitor cells in mouse [20] and human fetal heart [21]. *OCT4* expression may thus not be obligatory in adult cardiac stem/progenitor cells.

It should be acknowledged that in the present study, only biopsy samples from patients undergoing Maze surgery against atrial fibrillation were studied. We can not exclude that this might have affected cell distribution or commitment within the C-kit+CD45- population. However, this was the only possible source of tissue samples that permitted comparison between right and left atrial biopsies and yielded enough material for single-cell sorting. Furthermore, atrial fibrillation is not directly associated with ischemia which theoretically could have explained the predominant endothelial commitment observed in the present study.

Another limitation of the current study is lack of protein expression data. Unfortunately, it is currently not technically possible to analyze protein expression of single-cells with the same degree of specificity and sensitivity as gene expression by qPCR. Immuno PCR development show promising results [22] and may in future make this possible, also for single-cell applications.

In conclusions, we show that only right atrium contained a clear population of C-kit+CD45- cells. This population was heterogeneous in regard of cardiac and endothelial differentiation. While most cells expressed markers for endothelial genes, a minor portion was found to express cardiac genes. This indicates that instead of being truly multipotent, the C-kit+CD45- population is heterogeneous and consists of already lineage committed progenitors.

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Disclosures

None declared

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Legends to figures

Fig. 1

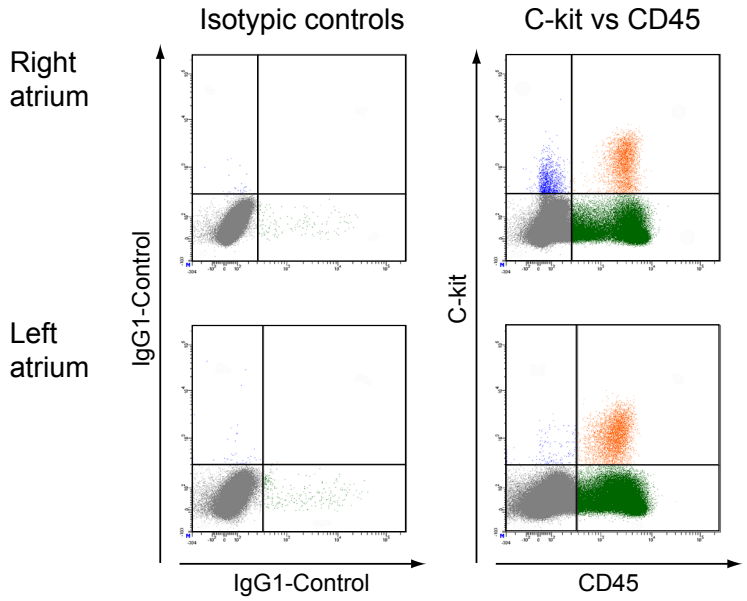
FACS analysis of C-kit and CD45 in cells from right and left atrial biopsies. Plots to the left show gating strategy for one representative experiment while statistic tables to the right show mean value for all analyzed biopsies ± SEM. Numbers (n) of biopsies analyzed are indicated in the figure. * denotes a significant difference ($P < 0.05$) in expression between right and left atrium.

Fig. 2

Cell and gene cluster analysis of single-cell sorted C-kit+CD45- cells with either *CKIT*+ VWF- or *NKX2.5*+ gene expression (n = 71 cells).

Cluster analysis was carried out according to Wards algorithm, Euclidean distance. Clustering of genes is shown on the X-axis whereas clustering of cells is shown on the Y-axis. Arrows denotes *NKX2.5*+ cells also positive for *VWF*.

Fig 1



C-kit- CD45-	89	± 2	%
C-kit+ CD45-	*0.078	± 0.018	%
C-kit+ CD45+	*0.42	± 0.09	%
C-kit- CD45+	7.7	± 0.6	%

n = 7

C-kit- CD45-	87	± 3	%
C-kit+ CD45-	*0.010	± 0.004	%
C-kit+ CD45+	*0.86	± 0.12	%
C-kit- CD45+	9.5	± 3.6	%

n = 6

Fig 2

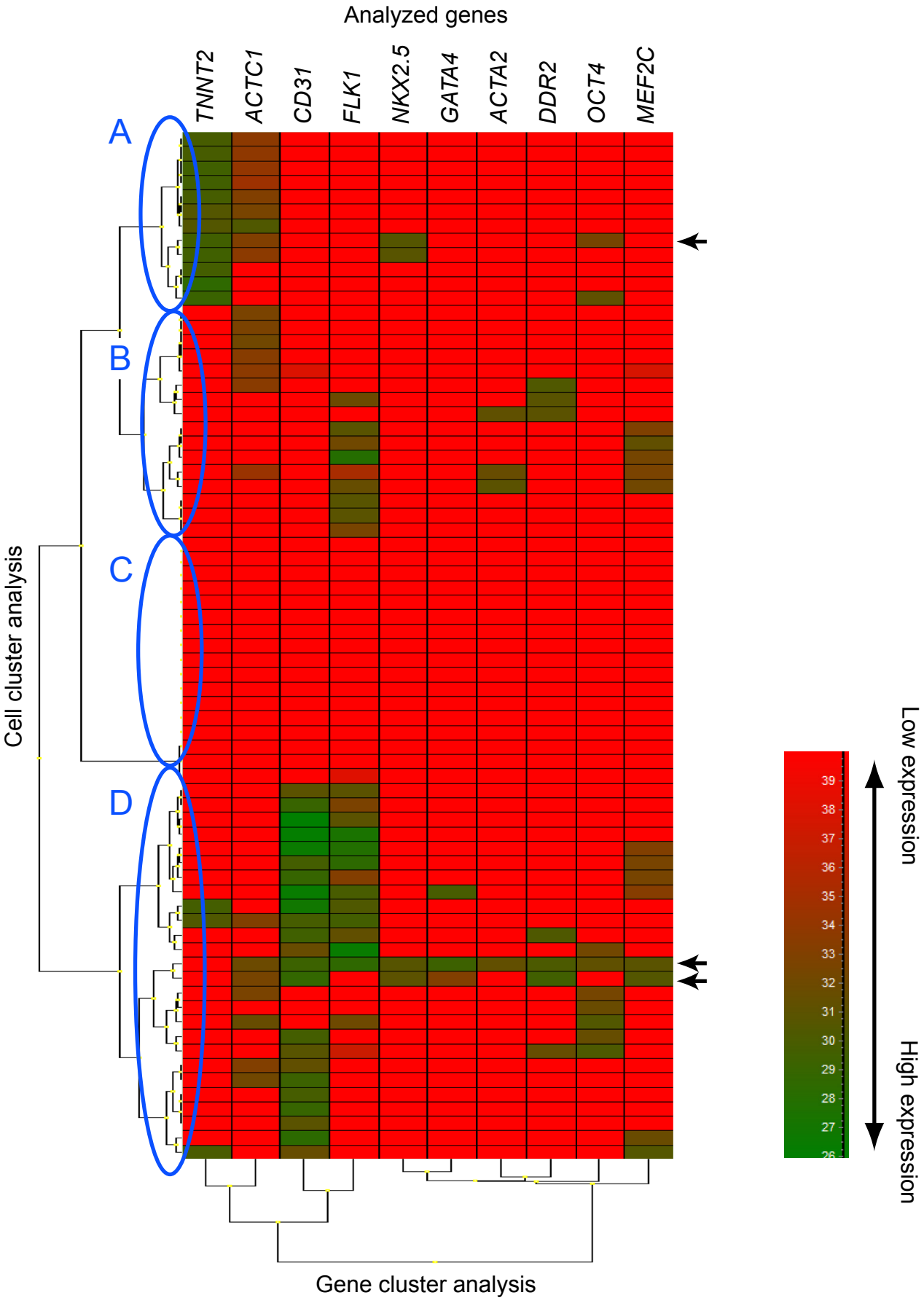

Analyzed genes
TNNT2
ACTC1
CD31
FLK1
NKX2.5
GATA4
ACTA2
DDR2
OCT4
MEF2C
A
B
C
D
Cell cluster analysis
Gene cluster analysis
39
38
37
36
35
34
33
32
31
30
29
28
27
26
Low expression
High expression

Table 1. Initial gene expression characterization of all CKIT+ cells

Gene	Percentage of all *CKIT*+ cells
VWF	81. %
FLK1	72. %
NKX2.5	1.1 %
CD45	0.0 %

C-kit+ cells were single cell sorted and first analyzed for CKIT gene expression to confirm C-kit+ identity. All 354 cells expressing CKIT were then further analyzed for endothelial, cardiac and hematopoietic markers and percentages of positive cells were calculated as shown above.

Electronic Supplemental Material

Article title: Human C-kit+CD45- cardiac stem cells are heterogeneous and display both cardiac and endothelial commitment by single-cell qPCR analysis

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Expanded methods

Cell isolation procedure

Biopsies were collected in cold phosphate buffered saline (PBS) and washed with additional PBS to remove residual blood, weighted and cut into small pieces before enzymatic digestion. Tissue pieces were digested with Liberase type TM 0.56 U/ml (Roche, Basel, Switzerland) in DMEM:F12 (Invitrogen, Carlsbad, CA, USA) at 37°C for 4.5 hours with magnetic stirring, washed once and further incubated for 10 minutes in 0.05% Tryspin-EDTA (Invitrogen). The obtained cells were resuspended in DMEM:F12 supplemented with 10% fetal bovine serum (FBS, Sigma St. Louis, MO, USA) and filtered through a 100 µm cell strainer (BD, Franklin Lakes, NJ, USA) to remove residual tissue fragments and cardiomyocytes. The remaining cell suspension was then subjected to an epitope regeneration step in order to enhance detection of C-kit expression. Cells were allowed to regenerate their epitopes for 7 - 10 hours in suspension culture dishes in DMEM:F12 supplemented with 5% FBS and 1mM EDTA (Sigma) with mild agitation. The cell suspension were then spun down and treated with an erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) for one minute at room temperature, then washed in cold FACS staining buffer (PBS supplemented with 5% FBS, 1% BSA (Sigma) and 2 mM EDTA) and subjected to flow cytometry analysis.

Single cell qPCR analysis

Cells were directly sorted to 5 µl Lysis solution with 0.5 µl DNase1 in a 96 well plate and incubated 5 min in room temperature before 1 µl stop mix (0.5 µl Stop solution/0.5 µl Lysis solution) was added per well. The plate was sealed and mixed with rotated hand movements, incubated 2 min at room temperature and stored at -80˚C. For cDNA synthesis, 12.5 µl 2x RT Buffer, 1.25 µl 20x RT Enzyme Mix and 6.25 µl RNase free water were added to each well.

Next, 2.5 µl of the cDNA were preamplified with 5 µl of the TaqMan preamp Master Mix (2x) and 2.5 µl of the pooled assay mix (0.2x). The preamplification was performed for 14 cycles. The preamplified cDNA was diluted 1:20 with RNase free water. 5 µl of the diluted cDNA was taken to each Gene Expression analysis with 4 µl RNase free water, 10 µl of the 2xTaqMan Master Mix and 1 µl of the specific gene assay per well.

Gene assays used for single cell qPCR

Gene	Assay ID (Life Technologies)
CREBBP	Hs00231733_m1
VWF	Hs00169795_m1
CD31	Hs00169777_m1
FLK1	Hs00176676_m1
CD45	Hs00236304_m1
C-KIT	Hs00174029_m1
NKX2.5	Hs00231763_m1
TNNT2	Hs00165960_m1
GATA4	Hs00171403_m1
ACTC1	Hs00606316_m1
OCT4	Hs01895061_u1
ACTA2	Hs00909449_m1
DDR2	Hs00178815_m1
MEF2C	Hs00231149_m1

Legends to Supplement figures

Supplement figure 1

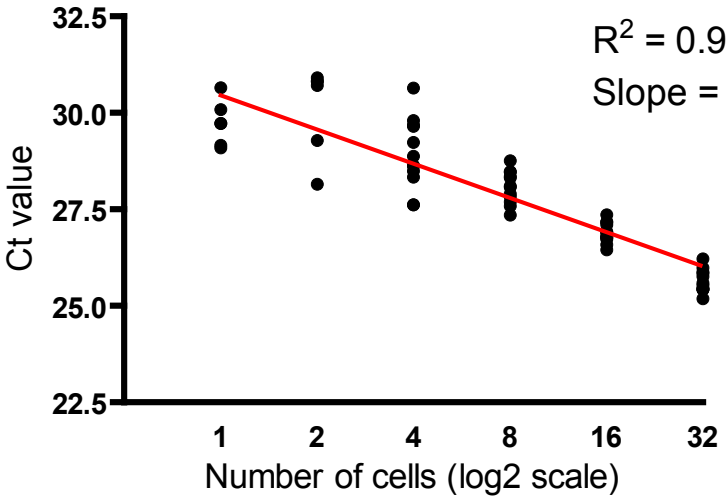
Fibroblasts were sorted by FACS in a dilution series at single cell mode. Cells were then analyzed for *CREBBP* expression. *CREBBP* is known to be stably expressed in large groups of cells and has previously been used as a reference gene[1].

Notably, variation in expression increased when lower number of cells was sorted. This would be expected since the inter-cellular variation in gene expression becomes more markedly the fewer cells that are analyzed. Notably, when 1 and 2 cells were sorted, about half of the cells had undetectable levels of *CREBBP*. This could either be due to cyclic variation in expression or due to technical reasons (i.e. cells were sorted slightly off centre and was not lysed properly). Linear regression was carried out as indicated in the figure. Slope was demined to -0.89, quite close to the theoretically expected value of -1.

Supplemental references

1. Synnergren J, Giesler TL, Adak S, Tandon R, Noaksson K, Lindahl A, Nilsson P, Nelson D, Olsson B, Englund MC, Abbot S, Sartipy P. Differentiating human embryonic stem cells express a unique housekeeping gene signature. *Stem Cells*. 2007;25:473-480

Suppl. Fig 1



R^2 = 0.93 (calculated on average values)

Slope = -0.89