Higher frequencies of BCRP$^+$ cardiac resident cells in ischaemic human myocardium

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Aims	Several cardiac resident progenitor cell types have been reported for the adult mammalian heart. Here we characterize their frequencies and distribution pattern in non-ischaemic human myocardial tissue and after ischaemic events.
Methods and results	We obtained 55 biopsy samples from human atria and ventricles and used immunohistological analysis to investigate two cardiac cell types, characterized by the expression of breast cancer resistance protein (BCRP)/ABCG2 [for side population (SP) cells] or c-kit. Highest frequencies of BCRP$^+$ cells were detected in the ischaemic right atria with a median of 5.40% (range: 2.48–11.1%) vs. 4.40% (1.79–7.75%) in the non-ischaemic right atria ($P = 0.47$). Significantly higher amounts were identified in ischaemic compared with non-ischaemic ventricles, viz. 5.44% (3.24–9.30%) vs. 0.74% (0–5.23%) ($P = 0.016$). Few numbers of BCRP$^+$ cells co-expressed the cardiac markers titin, sarcomeric α-actinin, or Nkx2.5; no co-expression of BCRP and progenitor cell marker Sca-1 or pluripotency markers Oct-3/4, SSEA-3, and SSEA-4 was detected. C-kit$^+$ cells displayed higher frequencies in ischaemic (ratio: 1:25 000 ± 2500 of cell counts) vs. non-ischaemic myocardium (1:105 000 ± 43 000). Breast cancer resistance protein$^+$/c-kit$^+$ cells were not identified. Following *in vitro* differentiation, BCRP$^+$ cells isolated from human heart biopsy samples ($n = 6$) showed expression of cardiac troponin T and α-myosin heavy-chain, but no full differentiation into functional beating cardiomyocytes was observed.
Conclusion	We were able to demonstrate that BCRP$^+$/CD31$^-$ cells are more abundant in the heart than their c-kit$^+$ counterparts. In the non-ischaemic hearts, they are preferentially located in the atria. Following ischaemia, their numbers are elevated significantly. Our data might provide a valuable snapshot at potential progenitor cells after acute ischaemia *in vivo*, and mapping of these easily accessible cells may influence future cell therapeutic strategies.
Keywords	Cardiac resident progenitor cells • Myocardial restoration • Ischaemic heart disease • Heart failure

Introduction

Stem cell-based therapies might be therapeutic alternatives for the restoration of myocardial function after damage or disease, which are highly desirable as the heart lacks significant endogenous regenerative potential.[1,2] With increasing knowledge of stem cell-based therapies, new hope has arisen for the treatment of heart failure. Various stem cell types are undergoing evaluation including haematopoietic stem cells,[3] endothelial progenitors,[4] mesenchymal stem cells (MSCs),[5] and the so-called SP cells.[6,7] Furthermore, skeletal myoblasts[8] as well as embryonic stem cells (ESC)[9] or induced pluripotent stem cells (iPS)[10,11] are being considered. However, the lack of efficacy, non-specific differentiation, clinical safety issues as well as the ethical debate over ESC still limit clinical application and underline the demand for cardiac-specific stem cell types.

Recent data indicate that the heart hosts its own stem/progenitor cell populations[12–14] with the capacity to differentiate along all cardiac cell lineages.[13,15,16] These cardiac resident progenitor cells (CRPC) include primitive cells expressing the stem-cell-factor

receptor c-kit (CD117)[16], and Sca-1$^+$ cells detected in mice[12] and in humans.[17] Despite their frequent denomination as stem cells, not all these cell types were demonstrated to fulfil the criteria of *bona fide* stem cells as being self-renewing, clonogenic, and multipotent.[18] Martin *et al.* detected cardiac side population (cSP) cells in the heart.[19] Side population cells can be isolated by dual-wavelength flow cytometry because of their capacity to efflux Hoechst dye, a process mediated by the ATP-binding cassette transporter breast cancer resistance protein (BCRP).[6,7] Breast cancer resistance protein (also known as ABCG2) has been recently reported to play a functional role in modulating the proliferation, differentiation, and survival of cSP cells,[20] but is also present within endothelial cells of the human heart.[21] Another CRPC population was isolated by Messina *et al.*[22] from murine and human hearts. They were the first to demonstrate extensive *in vitro* proliferation of the isolated cells in so-called cardiosphere cultures. *In vitro* cultivation of CRPC might be a decisive step forward for future myocardial restoration requiring large cell numbers.

The expression pattern and frequency of CRPC in a healthy or diseased human heart has not been explored so far. Herein we identify and quantify of human BCRP$^+$ and c-kit$^+$ cells in the different compartments of the heart and ischaemic vs. non-ischaemic myocardium, and assess whether human BCRP$^+$ cells can be considered cardiac progenitor cells. The detailed clarification of the distribution and frequencies of CRPC in ischaemic and non-ischaemic heart samples might have an important impact on effective stem cell treatment strategies in the future.

Methods

Patient demographics

After informed consent, 55 tissue-samples were obtained from 50 patients (male = 32/female = 18). The patients were Caucasians (17–83 years) and suffered either from ischaemic heart disease (n = 33) or non-ischaemic heart-diseases, including valve-disease (n = 13), non-ischaemic, dilative cardiomyopathy (n = 2), and congenital heart disease (n = 2). Except three emergency cases including two left ventricular assist device (LVAD) implantations due to ischaemic cardiomyopathy (n = 2) and one coronary artery bypass grafting (CABG) for instable angina (n = 1) within the ischaemic group, and two heart transplantations due to dilated cardiomyopathy (n = 2) in the non-ischaemic group, all the patients were elective cases. The treatment included aspirin, angiotensin-converting enzyme inhibitors, beta-blockers, and others. The demographics are summarized in *Table 1*.

Tissue samples

Tissue samples sized 5 × 5 mm were harvested from all areas of the heart. They were obtained from the right atrium (n = 36) after cannulation during coronary artery bypass grafting (CABG). Endomyocardial biopsy samples of the left atrium (n = 4), left ventricle (n = 10), and right ventricle (n = 5) were taken during CABG and all other open heart procedures. For ischaemic patients, after careful intra-operative macroscopic evaluation, biopsy samples were harvested from the border zone of the ischaemic/infarcted area of the left ventricle, but not directly from the infarcted area. The samples were fixed in 4% paraformaldehyde for 2 h following 24 h in 30% sucrose, embedded in TissueTek (Sakura-Finetek), frozen in liquid nitrogen, and cut into 6-μm sections.

Table 1 Patient demographics

$n = 50$	Ischaemic ($n = 33$)	Non-ischaemic ($n = 17$)
Male	26	6
Female	7	11
Height (cm)	171 ± 7	172 ± 7
Weight (kg)	77 ± 12	80 ± 7
BMI	26 ± 4	27 ± 3
EuroScore	4 ± 3	7 ± 4
Ejection fraction	56 ± 13	53 ± 18
Blood pressure systolic (mmHg)	134 ± 24	136 ± 20
Blood pressure diastolic (mmHg)	71 ± 17	75 ± 11
Smoker (%)	17 (52)	14 (82)
Positive family history of any cardiac disease (%)	15 (45)	11 (64)
Diabetes mellitus (%)	9 (27)	5 (29)
Hypertension (%)	14 (42)	12 (70)
Dyslipidaemia (%)	20 (61)	13 (76)
Ischaemic heart disease (%)	33 (100)	0 (0)
Heart valve disease (%)	0 (0)	13 (76)
Dilative cardiomyopathy (%)	0 (0)	2 (12)
Congenital heart disease (%)	0 (0)	2 (12)
CABG (%)	23 (70)	0 (0)
Heart valve surgery (%)	0 (0)	13 (76)
Combined heart surgery (CABG + valve) (%)	8 (24)	0 (0)
Transplantation due to DCM (%)	0 (0)	2 (12)
LVAD implantation due to ICM (%)	2 (6)	0 (0)
Congenital heart surgery (%)	0 (0)	2 (12)
Aspirin (%)	27 (82)	16 (94)
Beta-blockers (%)	11 (33)	8 (47)
ACE inhibitors (%)	4 (12)	3 (18)

CABG, coronary artery bypass grafting; DCM, dilative cardiomyopathy; ICM, ischaemic cardiomyopathy; LVAD, left ventricular assist device.
All values are mean (± SD).

Immunostaining and cell counts

For a quantitative analysis of progenitor cell frequencies, immunostaining was performed in at least four sections of each tissue sample. The positive controls were as follows: BCRP$^+$ trophoblast cells in human placenta (see Supplementary material online, *Figure S1A*), c-kit$^+$ melanocytes in human skin (see Supplementary material online, *Figure S1B*), c-kit$^+$/tryptase$^+$ mast cells in inflammatory skin lesions (see Supplementary material online, *Figure S1C–F*), and titin$^+$ cells and CD31$^+$ endothelial cells in human cardiac tissue. For pluripotent stem cell markers Oct-3/4, SSEA-3, and SSEA-4, human-induced pluripotent stem cell colonies served as positive controls (see Supplementary material online, *Figure S2*). Staining procedures and antibodies used are described in detail in the Supplementary material online, Methods.

Analysis of four slides per tissue sample was performed. At least 12 randomized images per slide were collected with an Olympus-IX81 fluorescence-microscope using a F-View-II camera and the Olympus-analysisD® software (Olympus; Tokyo, Japan). Images were analysed with the ImageJ® software and the BCRP$^+$ and c-kit$^+$ cell counts were calculated in at least 12 randomized microscopic fields per slide. We discriminated between BCRP$^+$/CD31$^+$ endothelial cells and BCRP$^+$/CD31$^-$ cells. Similarly, we discriminated between c-kit$^+$/mast-cell-tryptase$^+$ mast cells and c-kit$^+$/tryptase$^-$ resident progenitor cells.

Isolation of breast cancer resistance protein$^+$ cells from adult human heart samples

Breast cancer resistance protein$^+$ cells were isolated from human cardiac tissue samples using collagenase B and dispase II as described by Pfister *et al.*[23] These procedures are described in detail in the Supplementary material online, Methods.

In vitro differentiation of breast cancer resistance protein$^+$ cells

The cells were seeded into 24well plates (Nunc/Thermo Fisher Scientific) after coating with Matrigel (1:30, BD Biosciences, Heidelberg, Germany), and the cell culture medium consisted of 80% IMDM (with Glutamax), 20% foetal calf serum (HyClone/Thermo Fisher Scientific, Bonn, Germany), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, 100 μg/mL penicillin, and 100 μg/mL streptomycin (from PAA Laboratories, Coelbe, Germany). Twenty-four hours after seeding, the cells were treated with 100 nM oxytocin (Sigma-Aldrich, Munich, Germany) for 72 h as described by Oyama *et al.*[24]

Gene expression analysis

Quantitative real-time PCR was used to determine the gene expression of BCRP$^+$ cells after *in vitro* differentiation. These procedures are described in detail in the Supplementary material online, Methods.

Statistical analysis

Descriptive data are presented as mean ± standard deviation or median with range where appropriate. For statistical analysis using SPSS 18 (SPSS, Inc., Chicago, IL, USA), the Mann−Whitney test was performed. To avoid biased estimates for patients that provided more than one cardiac sample ($n = 3$), medians of samples within patients were computed and a patient-specific ($n = 50$) Mann−Whitney test was performed. Significance was evaluated by two-tailed testing and assumed to be $P < 0.05$.

Results

Morphological assessment of putative progenitor cells detected in the heart

Breast cancer resistance protein$^+$/CD31$^-$ cells were identified in all areas of the heart, appearing mostly small and oval shaped. They were distributed evenly over the whole microscopic field (*Figure 1A−C*). The major part of these cells did not stain positive for the cardiac marker titin, but were located either between or in close relation to the surrounding cardiomyocytes as well as in the interstitial space. However, also titin-positive BCRP$^+$/CD31$^-$ cardiac progenitors could be found, albeit in low numbers (*Figure 1D−F*). Some BCRP$^+$/CD31$^-$ cells stained positive for sarcomeric α-actinin, or the earlier cardiac marker Nkx2.5 for the identification of cardiac progenitor cells (see Supplementary

material online, *Figures S3* and *S4*). In BCRP$^+$/CD31$^-$ cells, we did not detect staining for the progenitor cell marker Sca-1 (see Supplementary material online, *Figure S5*) or pluripotent stem cell markers Oct-3/4, SSEA-3, and SSEA-4 (see Supplementary material online, *Figures S6* and *S7*). Single cells expressing Sca-1 and Oct-3/4 were observed within the myocardium (see Supplementary material online, *Figures S5* and *S6*). Cells expressing SSEA-3 or SSEA-4 were detected in vessels and single cells staining positive for CD31 (see Supplementary material online, *Figure S7*); however, we did not analyse these cells further.

Breast cancer resistance protein$^+$/CD31$^+$ cells were also detected in all areas, representing endothelial cells with the typical elongated morphology. They were characteristically located in small capillaries and in the inner wall of arterioles (*Figure 1G−I*).

Resident c-kit$^+$ cells were detected both in the ventricles and in the right atrium. These cells were small and round shaped, without the typical cardiomyocyte morphology, and did not stain positive for cardiac markers (*Figure 2A−C*).

Distribution and frequencies

Breast cancer resistance protein$^+$/CD31$^-$ cells were found more frequently in ischaemic hearts ($n = 33$) vs. non-ischaemic ($n = 22$) hearts with a median of 5.38% (range: 2.48−11.1%) vs. 3.57% (0−7.75%) ($P = 0.026$) (*Figure 3A*). In the atria, the highest frequency of BCRP$^+$/CD31$^-$ cells was detected in the right atria of the ischaemic group ($n = 26$) with a median of 5.40% (2.48−11.10%) vs. 4.40% (1.79−7.75%) ($P = 0.47$) in the non-ischaemic group ($n = 10$) (*Figure 3B*). Also in the ventricles, a significantly increased number of BCRP$^+$/CD31$^-$ cells was detected in ischaemic ventricles ($n = 6$) compared with non-ischaemic ventricles ($n = 9$) with a median of 5.44% (3.24−9.30%) vs. 0.74% (0−5.23%), respectively ($P = 0.016$) (*Figure 3C*).

The frequency of BCRP$^+$/CD31$^-$ cells was analysed with respect to age and gender. In both the groups (>65 and ≤65 years), BCRP$^+$/CD31$^-$ cells were found more frequently in ischaemic hearts vs. non-ischaemic hearts (data not shown). Interestingly, the number of BCRP$^+$/CD31$^-$ cells tended to be higher in the age group >65 years than for patients ≤65 years. This was found for the ischaemic group with 5.79% (2.48−11.10%) BCRP$^+$/CD31$^-$ cells ($n = 21$) vs. 4.74% (2.51−7.35%) ($n = 12$), respectively ($P = 0.24$). Similarly, in the non-ischaemic samples, the number of BCRP$^+$/CD31$^-$ cells was 4.17% (3.57−6.23%) ($n = 7$) in patients >65 years vs. 1.63% (0−7.75%) ($n = 15$) in patients ≤65 years ($P = 0.20$).

Analysing ischaemic samples from all parts of the heart, we found a slight tendency towards a higher number of BCRP$^+$/CD31$^-$ cells in female patients than in male patients, viz. 5.42% (3.54−11.10%) ($n = 7$) vs. 5.01% (2.48−9.64%) ($n = 26$); however, this effect did not reach statistical significance ($P = 0.35$).

Breast cancer resistance protein$^+$/CD31$^-$ cells were analysed for cardiac differentiation in the heart to investigate whether they can be considered progenitor cells. In 50% of all tissue-samples, some BCRP$^+$/CD31$^-$ cells stained positive for the cardiac marker titin, albeit in low numbers [median of 0.36% (0−1.27%) in the ischaemic heart vs. 0.18% (0−0.39%) in the non-ischaemic heart ($P = 0.013$) (*Figure 4A*)]. The distribution of BCRP$^+$/CD31$^-$/titin$^+$ cells was identified as follows: 0.33% (0−0.92%) of cells were
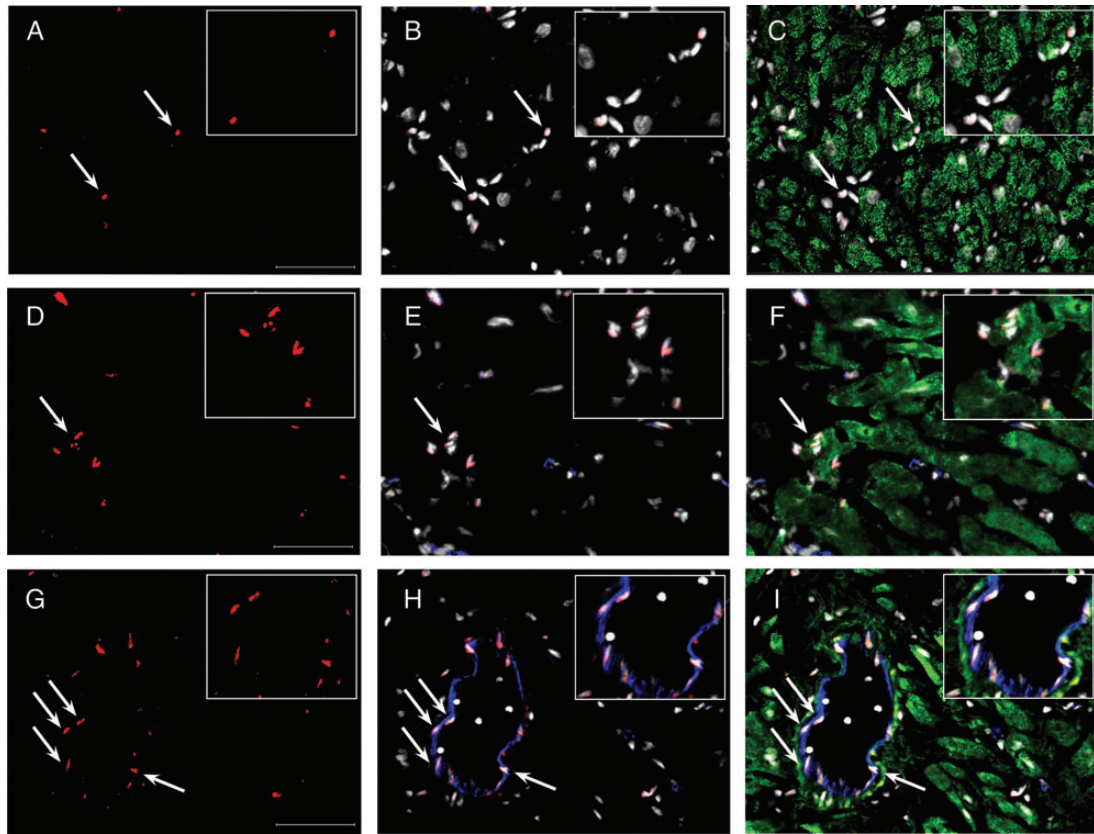
Figure 1 Breast cancer resistance protein$^+$ cells in the adult human heart. Cryosections of heart tissue were stained with antibodies to breast cancer resistance protein (red), titin (green), and CD31 (blue). Nuclei were stained with DAPI (white) ($\times$40). Inset shows cells pointed out by arrows. (A–C) (right atrium/♀/61 years): breast cancer resistance protein$^+$/CD31$^-$ cells (arrows) presumably representing resident cardiac progenitors were identified in all areas of the heart. (D–F) (right atrium/♂/70 years): A subset of breast cancer resistance protein$^+$/CD31$^-$ cells (arrows) stain also positive for the cardiac marker titin (here: 0.50% of total cell numbers). (G–I) (right atrium/♂/83 years): breast cancer resistance protein$^+$/CD31$^+$ cells (arrows) representing endothelial cells could be detected in all areas of the heart, mostly located in small capillaries and arterioles. Scale bar: 50 μm.
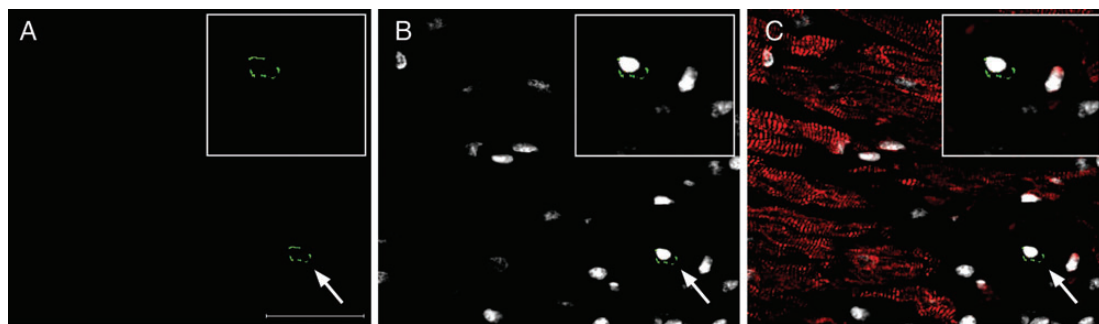

Figure 2 C-kit$^+$ cells in ischaemic human myocardium. Cryosections of adult human heart tissue were stained with antibodies to c-kit (green, indicated by arrows), titin (red), and mast cell tryptase (blue). Nuclei were stained with DAPI (white). Inset shows cells pointed out by arrows. (A–C) Tissue sample (left ventricle/♂/75 years) after myocardial infarction; very low frequencies of c-kit$^+$/ mast cell tryptase$^-$ cells were detected (arrow), while no mast cells were observed. Scale bar: 50 μm.
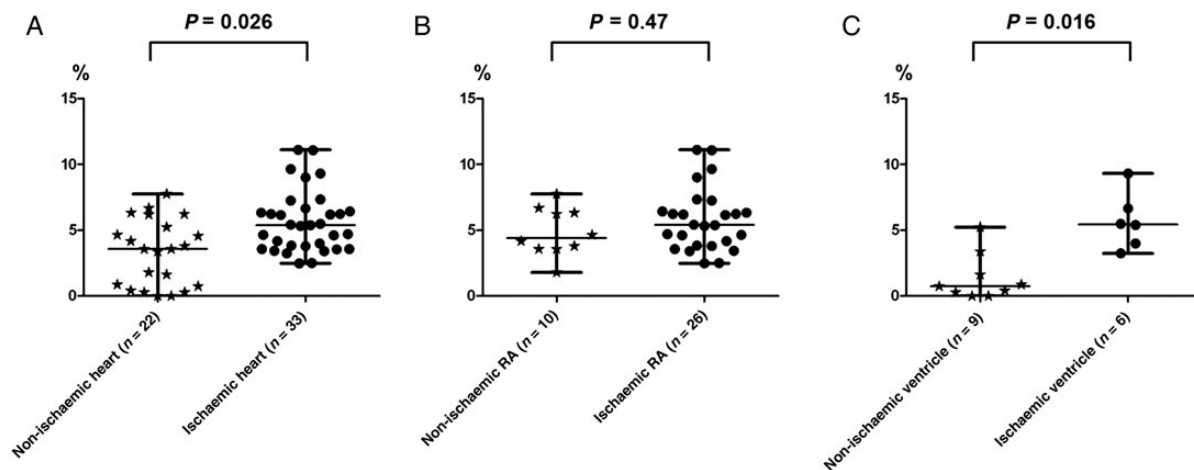
Figure 3 Higher frequency of cardiac resident breast cancer resistance protein$^+$/CD31$^-$ cells in the ischaemic human heart. Distribution and frequencies (percentage of all cardiac cells) of all breast cancer resistance protein$^+$/CD31$^-$ cells in non-ischaemic vs. ischaemic hearts (A), non-ischaemic vs. ischaemic atria (B) as well as non-ischaemic vs. ischaemic ventricles (C). Graphs are shown with median and range. To avoid biased estimates for patients that provided more than one cardiac sample, a patient-specific Mann−Whitney test was performed. RA, right atrium.
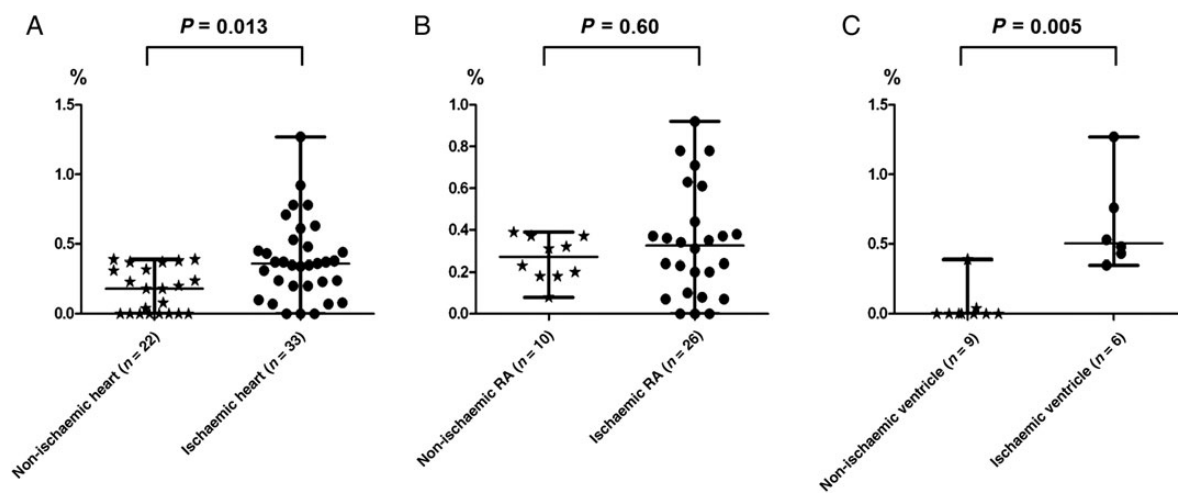

Figure 4 Higher frequency of Titin$^+$ subsets of breast cancer resistance protein$^+$/CD31$^-$ cardiac resident progenitor cells in the ischaemic human heart. Distribution and frequencies (percentage of all cardiac cells) of breast cancer resistance protein$^+$/CD31$^-$/Titin$^+$ cells in non-ischaemic vs. ischaemic hearts (A), non-ischaemic vs. ischaemic atria (B) as well as non-ischaemic vs. ischaemic ventricles (C). Graphs are shown with median and range. In order to avoid biased estimates for patients that provided more than one cardiac sample, a patient specific Mann−Whitney test was performed. RA, right atrium.

detected in the ischaemic right atria ($n = 26$) vs. 0.27% (0.08−0.39%) in non-ischaemic right atria ($n = 10$) ($P = 0.60$) (*Figure 4B*). Higher BCRP$^+$/CD31$^-$/titin$^+$ cell numbers were detected in ischaemic ventricles ($n = 6$) compared with non-ischaemic ventricles ($n = 9$) [median of 0.50% (0.35−1.27%) vs. 0% (0−0.39%; $P = 0.005$) (*Figure 4C*].

Breast cancer resistance protein$^+$/CD31$^+$ cells, representing endothelial cells, were also found more frequently in ischaemic hearts compared with non-ischaemic hearts [median of 5.50% (0.91−11.98%) vs. 3.14% (0−8.33%); $P = 0.04$] (see Supplementary material online, *Figure S8*). In detail, the distribution pattern of BCRP$^+$/CD31$^+$ cells was identified as follows: 7.37% (5.51−11.09%) BCRP$^+$/CD31$^+$ cells were detected in ischaemic ventricles vs. 2.18% (0−6.89%) in non-ischaemic ventricles ($P = 0.01$). In contrast to this, the right atria showed almost the same amount of BCRP$^+$/CD31$^+$ cells in the ischaemic ($n = 26$) and

non-ischaemic groups ($n = 10$) [5.27% (0.91–11.98%) vs. 4.53% (2.17–8.33%); $P = 0.60$].

C-kit+ cells were detected in 30% of the samples and were found in higher numbers within ischaemic (ratio: 1:25 000 ± 2500 of cell counts) vs. non-ischaemic myocardium (1:105 000 ± 43 000) including ventricles and the right atrium. A detailed comparison between ischaemic and non-ischaemic ventricles showed an increased frequency of c-kit+ cells within ischaemic ventricles (1:25 000 ± 2500) vs. either no or only very low numbers of c-kit+ cells (1:120 000 ± 50 000) within non-ischaemic ventricles. The cell ratio in ischaemic ventricles increased up to five-fold ($P = 0.022$). Although BCRP+ and c-kit+ cells were found in close proximity (*Figure 5*), no BCRP+/c-kit+ double-positive cells were detected.

Isolation and *in vitro* differentiation of breast cancer resistance protein+ cells

Using immunomagnetic bead separation, cells were enriched from dissociated human heart samples ($n = 6$) on the basis of their expression of the surface marker BCRP. Co-purified adult human cardiomyocytes did not adhere to the cell culture dishes (see Supplementary material online, *Figure S9A*) and were removed upon medium exchange. Breast cancer resistance protein+ and BCRP− cells were cultivated on matrigel-coated plates in differentiation medium. Breast cancer resistance protein− cell fractions showed higher proliferation rates than BCRP+ cells and were frequently overgrown with fibroblast-like cells during cultivation (see Supplementary material online, *Figure S9B–D*). To test for differentiation potential as described by Oyama et al.[24] in the rat model, the cells were treated with 100 nM oxytocin for 72 h. However, no spontaneous beating of isolated cells was observed during cultivation for 21 days (*Figure 6A–C*).

After 21 days, no cTnT expression was detected in BCRP− cells by immunostaining (*Figure 6D*). In contrast, the majority of BCRP+ cells stained positive for cTnT (*Figure 6E* and *F*), with a diffuse and/or a punctuate dotted linear staining pattern (*Figure 6F*). Interestingly, BCRP+ cells not treated with oxytocin seemed to display a similar differentiation capacity, a direct comparison of oxytocin-treated with untreated cells is given in Supplementary material online, *Figure S10*. On Day 21, quantitative real-time PCR detected an 8.3-fold (± 2.4) increase in α-myosin heavy chain expression in oxytocin-treated BCRP+ cells vs. untreated BCRP+ cells (see Supplementary material online, *Figure S10E*). However, it has to be noted that the small sample number and size did not allow statistical analysis or the evaluation of other markers of cardiac differentiation.

Discussion

It was proposed that the cardiac SP cell population functions as a progenitor cell population for the development, maintenance, and
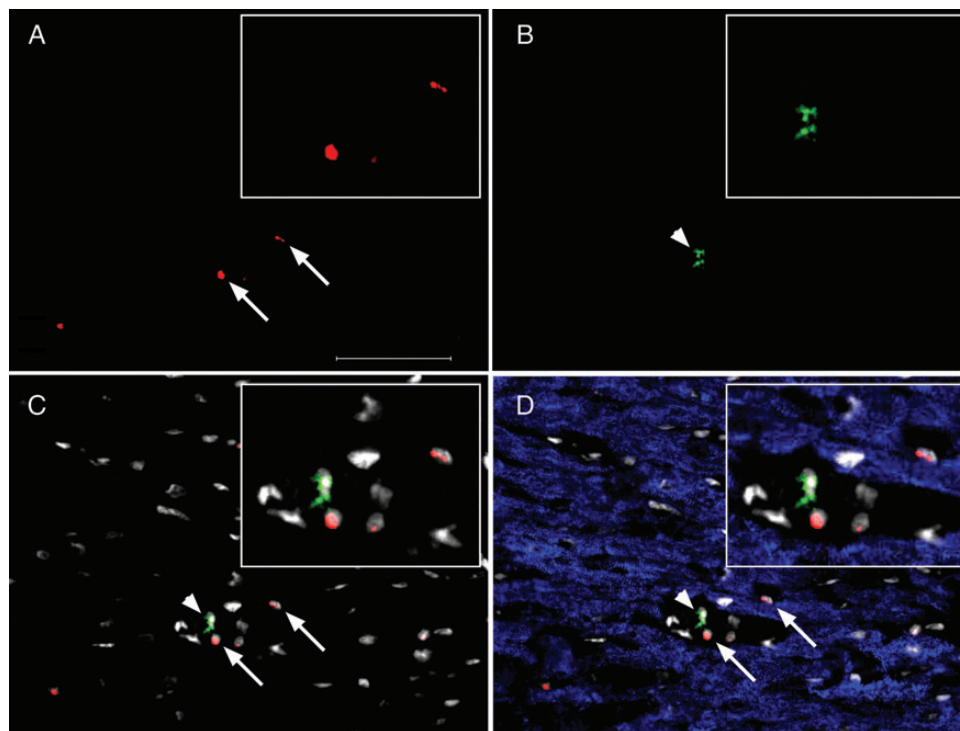

Figure 5 No c-kit/breast cancer resistance protein double-positive cells were detected in the adult human heart. Tissue sample (left ventricle/♂/53 years) after myocardial infarction, cryosections were stained with antibodies to breast cancer resistance protein (red, indicated by arrows; *A, C, D*), c-kit (green, indicated by arrowheads; *B, C, D*), and titin (blue; *D*). Nuclei were stained with DAPI (white). Although breast cancer resistance protein+ and c-kit+ cells were found in close proximity (*D*), no breast cancer resistance protein+/c-kit+ double-positive cells were detected. Inset shows cells pointed out by arrows/arrowheads. Scale bar: 50 μm.
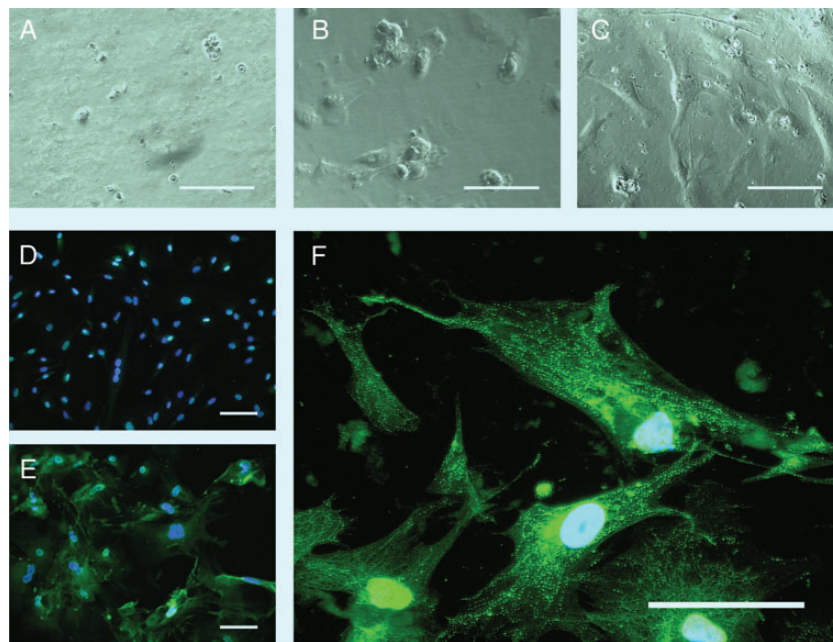
Figure 6 Breast cancer resistance protein^{+} cells isolated from the adult human heart differentiate *in vitro*. Morphology of oxytocin-treated breast cancer resistance protein^{+} cells on d3 (*A*), d14 (*B*), and d21 (*C*) of cultivation. After 21 days of cultivation, cells were fixed and stained for cardiac troponin T (cTnT, green). Nuclei were stained with DAPI (blue). While breast cancer resistance protein^{−} cells did not express cTnT (*D*), breast cancer resistance protein^{+} cells stained positive for cTnT (*E* and *F*) with punctuate dotted linear staining pattern, resembling pre-myofibrils, in oxytocin-treated cells (*F*). Scale bar: 100 μm.

repair of the heart.[19] Oyama *et al.*[24] were the first to demonstrate that cSP cells from post-natal rat hearts can differentiate into spontaneously beating cardiomyocytes after induction with non-toxic/-carcinogenic reagents such as oxytocin, as well as into other cell lineages, including endothelial cells and smooth muscle cells. These results support the concept that BCRP^{+}/CD31^{−} SP cells might be a possible source for regeneration.

Breast cancer resistance protein^{+} cells in the adult human heart

In this study, BCRP^{+}/CD31^{−} cells were detected in the atrial tissue of ischaemic but also in non-ischaemic patients at very similar levels. From a clinical point of view, the occurrence of CRPC even in non-ischaemic atria as described by Urbanek *et al.*[25] poses an excellent opportunity for a safe isolation during heart surgery or even by catheterization. Interestingly, there was a significant increase in the number of BCRP^{+}/CD31^{−} cells in ischaemic compared with non-ischaemic ventricle. These results might reflect repair mechanisms which are activated after ischaemic events. In the murine setting, Pfister *et al.*[26] found that *in vitro* cardiomyogenic differentiation was observed exclusively in cardiac SP cells lacking CD31. For the human heart, a potential activation and mobilization of these CRPC *in vivo* by a local cytokine application as proposed by Urbanek *et al.*[25] might be optimized by a detailed CRPC mapping. The localization of BCRP^{+} cells in non-ischaemic atria is in line with previous reports on the special role of the atria for c-kit^{+} cells, described as protected niches located in anatomical areas exposed to low levels of wall stress,[27] with higher

numbers of cardiac progenitor cells produced from right atrial tissue than obtained from other parts of the heart.[28]

Meissner *et al.*[21] reported that ABCG2 is variably expressed in endothelial cells of the human heart. In our study, BCRP^{+}/CD31^{+} endothelial cells[24,26] were detected in all areas of the heart within capillaries and small arterioles. Higher numbers were detected in ischaemic as well as in non-ischaemic atria and after myocardial infarction, increased numbers were identified in the ischaemic area of the ventricle, probably supporting angiogenesis. Only a few BCRP^{+}/CD31^{−} cells expressed the cardiac marker titin. Likewise, individual BCRP^{+}/CD31^{−} cells were found to express other cardiac markers such as sarcomeric α-actinin and Nkx2.5; however, their frequency was not quantified in this study. At the same time, the absence of expression of Oct-3/4, SSEA-3, and SSEA-4 in BCRP^{+} cells clearly indicates the lack of pluripotency. On the basis of these data, we assume that the BCRP[+] cell population described in this study might represent an already committed mesodermal progenitor state, as described by Ott *et al.*[29] As we did not detect human BCRP[+]/Sca-1^{+} cells, we conclude that the progenitor cell population described in our study is not identical to the Sca-1^{+} progenitor population found earlier in the human heart.[17] However, we cannot rule out heterogeneous Sca-1 expression in a subset of human BCRP^{+} cells, as was described for the mouse heart.[26,30]

C-kit^{+} cells in the adult human heart

Additionally, we were able to confirm the presence of c-kit[+] CRPC in the adult myocardium,[16,31] with higher frequencies (up

to five-fold) in the ischaemic ventricles and much lower numbers in the non-ischaemic myocardium. A comparison of progenitor cell frequencies observed in the human heart is difficult. Earlier reports used a variety of units, e.g. c-kit$^+$ cells per 100 mm^2,[15,16] c-kit$^+$ cells per cm^3 ($\times 10^3$)[25,32] (reviewed in,[33] there: cells per mm^3), or c-kit$^+$ cells as a percentage of the entire cell population.[34] The latter publication described a frequency of c-kit$^+$ cells of 1.1 $\pm$ 1.0% after enzymatic dissociation of $\sim$60 mg myocardial tissue ($n = 6$). Highest numbers of c-kit$^+$ cells with 8.9 $\pm$ 0.4% were reported for the right atrium of neonatal children <30 days of age.[35] Numbers of c-kit$^+$ cells found in our study in adult patients are significantly lower, with $\sim$0.004% in the non-ischaemic samples and 0.001% in the ischaemic samples. However, our data are in line with independent reports on frequencies of c-kit$^+$ cells of $\sim$0.002% in the adult human heart.[36] C-kit$^+$ resident cells did not stain positive for the cardiac marker titin. In compliance with the CRPC classification by Anversa et al.,[33] these cell types are considered rather primitive. Furthermore, we could show that there were no c-kit$^+$/BCRP$^+$ co-expressing stem/progenitor cell populations. This suggests that these two markers are expressed by two distinct cell populations in the human heart as was already proposed by Anversa et al.[33] for various species.[37]

In vitro differentiation of breast cancer resistance protein$^+$ cells isolated from the adult human heart

Although elevated numbers in ischaemic human heart might indicate a regenerative role for BCRP$^+$ cells, currently there is no proof of a direct link between progenitor cell numbers and myocardial restoration *in vivo*. As described for other stem/progenitor cell types, a variety of mechanisms is possible: The cells could (i) differentiate into tissue-specific cells, (ii) induce growth and differentiation through secreted factors, (iii) attract immune cells and affect their cytokine production, or (iv) secrete anti-apoptotic factors.[38] To investigate potential differentiation into cardiomyocytes, first we performed *in vitro* experiments using BCRP$^+$ cells isolated from human heart tissue samples. In contrast to reports from Oyama et al.[24] describing spontaneous beating of cardiomyocytes derived from rat SP cells, we did not observe beating of BCRP$^+$ cells isolated from human heart tissue at any time. This might be in line with reports by Yamahara et al.,[30] who did not find the generation of spontaneously beating cells from murine cardiac SP cells. It is currently unknown whether further optimized culture conditions for individual species and/or prolonged cultivation periods of >3 weeks could lead to full cardiac differentiation of human BCRP$^+$ cells. After cultivation for 21 days, we observed that a majority of BCRP$^+$ cells displayed diffuse staining of troponin T. Moreover, some cells showed periodically localized foci of intense labelling. While we did not detect a mature sarcomeric organization, this staining pattern might represent premyofibrils before lateral alignment,[39] as sometimes observed for early stages of cardiac differentiation in primate embryonic stem cells.[40] The interesting observation in our study that both oxytocin-treated and untreated BCRP$^+$ cells displayed troponin T expression might be explained by the fact that both were

cultivated in differentiation medium containing a distinct lot of foetal calf serum selected for efficient cardiac differentiation of human iPS cells. According to our quantitative PCR data, higher levels of α-myosin heavy chain (MHC) seem to be expressed in oxytocin-treated BCRP$^+$ cells. However, the small sample number and size did not allow statistical analysis. Although we cannot fully exclude the possibility that BCRP$^+$ cell cultures were contaminated with adult human cardiomyocytes, potentially contributing to α-MHC expression levels, such a contamination can be considered unlikely because of the absence of beating cells at any time. In our setting, troponin T staining in the majority of cells showed that human BCRP$^+$ cells can adopt some markers of immature cardiomyocytes *in vitro*. In contrast to rat cells,[24] however, we did not observe full differentiation into functional beating cardiomyocytes or other cardiac cell types. Therefore, it remains unclear whether they could represent cardiac progenitor cells in the human heart. This might argue for fundamental species differences with respect to differentiation potential and the physiological role of cardiac BCRP$^+$ progenitor cells, similar to differences in differentiation capacity observed for MSCs or cardiospheres.[22,41] Although the significantly increased number of those cells in the ischaemic myocardium strongly suggests an important role in regeneration processes, the formation of functional *de novo* myocardium from human cardiac BCRP$^+$ progenitor cells appears rather unlikely. Clearly, further studies will be needed to fully characterize the regenerative potential of this interesting cell population from the adult human heart.

Conclusion

In conclusion, we were able to demonstrate that BRCP$^+$/CD31$^-$ cells are more abundant in the human heart than their c-kit$^+$ counterparts. In the non-ischaemic heart, they are preferentially located in the atria. Following ischaemia, their numbers are increased significantly and, most interestingly, the highest change can be found in the left ventricle. At the same time, also the number of these BCRP$^+$ cells expressing the cardiac marker titin is highest in the left ventricle. Breast cancer resistance protein$^+$ cells could be isolated from the human heart and adopted certain markers of immature cardiomyocytes *in vitro*, but without differentiation into beating cells. Therefore, our data might provide a valuable snapshot at cardiac progenitor cells after acute ischaemia, even though absolute numbers of cells acquiring a myocardial phenotype are low and the overall impact on cardiac regeneration *in vivo* has to be investigated in the future.

Study limitations

In this proof of concept study, the patient cohort displayed certain heterogeneity, and only a minimal data set was collected for each patient with regard to demographic profiling. Therefore, in subsequent studies when including higher numbers of patients, it may be of value to correlate more specific patient characteristics (including complete medication) with the expression and frequencies of BCRP cells. Additionally, we restricted our study to c-kit$^+$ and BCRP$^+$ cells and did not evaluate the frequency and distribution of further cardiac stem and progenitor cell populations such as Sca-1$^+$ cells.

Clearly, expression of titin in a subpopulation of human BCRP$^+$ cells *in vivo* and expression of cardiac troponin T and α-MHC *in vitro* indicate that BCRP+ cells acquire certain myocardial characteristics *in vivo* and might have progenitor potential; however, it is uncertain whether human BCRP$^+$ cells can form functional beating cardiomyocytes similar to rat cells. In the case of c-kit$^+$ cells, the low numbers detected in our study might argue against a pivotal role in regenerative processes in the human heart. Furthermore, it has to be noted that the small number and size of myocardial tissue samples did not allow statistical analysis or the evaluation of other markers. Further investigation will be needed to thoroughly assess the role of human BCRP$^+$ and c-kit$^+$ cells in endogenous cardiac regeneration and their impact on regenerative therapies.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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