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ORIGINAL CONTRIBUTION

Pressure overload leads to an increase of cardiac resident stem cells

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Abstract Recent studies suggest that the mammalian heart possesses some capacity for cardiac regeneration. This regenerative capacity is primarily documented postnatally and after myocardial infarction or pressure overload. Although the cell type that mediates endogenous regeneration is unclear, cardiac stem cells might be considered as potential candidates. To determine the number of c-kit + cardiac resident cells under conditions of pressure overload, we evaluated specimens derived from $n = 8$ patients with pressure overloaded single right ventricles in comparison to $n = 4$ explanted hearts from patients with dilated cardiomyopathy and $n = 14$ biopsies from children after heart transplantation. The age of the patients ranged from 16 days to 19 years. For quantification of cardiac stem cells, c-kit+/mast cell tryptase-/CD45- cells were counted and expressed as percent of the total nuclei. In specimens from patients with dilated cardiomyopathy, 0.13 ± 0.09% c-kit +/mast cell tryptase-/CD45- cells were detected. However, in specimens from patients with pressure overloaded single right ventricles, the numbers of c-kit+/mast cell tryptase-/CD45- cells were significantly higher (0.41 ±0.24%, $p < 0.05$). Under conditions of pressure overload, the right ventricle shows an approximately three-fold increase in c-kit+/mast cell tryptase-/CD45- cardiac resident cells. Despite the fact that this increased number of c-kit+ cells is not sufficient to prevent the failing heart from congestive heart failure, understanding the mechanism that leads to an increase of presumably cardiac resident stem cells under conditions of pressure overload might help to develop new strategies to enhance endogenous repair.

Keywords Cardiac regeneration · c-kit · Cardiac stem cell · Pressure overload

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Introduction

Certain non-mammalian vertebrates possess a significant capacity for cardiac regeneration. Adult zebrafish, for example, can undergo complete cardiac regeneration without scar formation after ventricular muscle removal by surgical resection [28]. Recently, it was shown that cardiac regeneration also occurs postnatally in mammals. Surgical resection of the ventricular apex in 1-day-old mice stimulates a regenerative response that restores the damaged heart to its normal anatomy and function [27]. A number of studies have shown that the adult mammalian heart also possesses a measurable capacity for cardiomyocyte renewal [4, 10, 29]. However, this regenerative capacity is not sufficient to restore contractile function after substantial cardiac injury. Therefore, a scientific and clinical challenge is to stimulate the regenerative ability of the human heart.

Certain cell types might be used in order to achieve this goal. In contrast to the zebrafish heart, mammalian cardiomyocytes are withdrawn from the cell cycle and become binucleated. However, cell types that potentially regenerate cardiomyocytes are extracardiac stem and progenitor cells (for review see Ref. [2]), which especially in children were shown to repopulate the heart with around 0.5% newly regenerated cardiomyocytes per year [30]. These data provide evidence that stem cells or precursor cells contribute to the replacement of adult mammalian cardiomyocytes during normal aging.

Also cardiac resident stem cells (for review see Ref. [16]) were reported to contribute to regeneration of cardiomyocytes. It was shown that intracoronary injection of cardiac resident stem cells improved ventricular function in an animal model [18]. Furthermore cardiac stem cells were shown to improve ventricular function in patients with ischaemic cardiomyopathy significantly [5,8]. Cardiac resident stem cells are defined as lineage negative cells that do not express transcription factors or membrane and cytoplasmic proteins shared by mature cell types. Markers used to define these lineage negative cells with a very high nucleus/cytoplasm ratio are c-kit, Sca-1, and MDR-1 [9, 10, 20, 22]. Cardiac lineage negative c-kit+ cells possess the three properties of stemness: clonogenicity, self-renewal, and multipotentiality [3]. CSC was first described to accumulate in the atria of the right ventricle compared to the left atrium or the ventricles. Another study also outlined the superiority of the right atrium for CSC; however, they found more CSC in the left ventricle than in the right ventricle [11]. Because of the limited number of samples in this study from the right ventricle ($n = 2$), the statistical relevance was questioned [26]. CPCs are most abundant in the neonatal period and rapidly decrease over time [1], [23]. While Mishra et al. [23] reported that c-kit+ cells declined from 8.9% in neonates to 3.2% in children >2 years of age in the right atrium, Amir et al. [1] reported values from 0.4% at birth falling to around 0.1% at the end of the first months after birth in the right ventricle.

It is known that pressure overload by transverse aortic constriction induced a significant formation of new cardiomyocytes in mice [10, 24]. Furthermore, pulmonary artery banding stimulates the right ventricle and changes regulation of several genes [12], and administration of angiotensin II leads to an increase in blood pressure and a following increase of c-kit+ cells [32]. Guided by these observations, we aimed to determine the amount of cardiac resident stem cells in explanted hearts of children with univentricular hearts and chronic pressure overload.

Methods

Specimen selection

The heart transplantation database of the pediatric heart center in Giessen was screened and 12 samples from explanted hearts were chosen. The underlying diagnoses were univentricular heart from the right ventricular type in eight cases and dilated cardiomyopathy in four cases. Hearts were excluded, when patients were dependent on cardiac assist devices or patients suffered from sepsis. Furthermore, 14 biopsies of heart-transplanted children were analysed. In these cases, rejection was evaluated histologically and only biopsies with rejection grade ISHLT 0 or 1A were evaluated. Endomyocardial biopsies were taken according to the post-transplantation evaluation programme in the pediatric heart transplantation center in Giessen. Approval for the study of human tissue was granted by the Ethics Committee of the University of Giessen and Marburg and complied with the Declaration of Helsinki.

Immunohistochemistry

According to the standard procedures, 5-µm sections were prepared from formalin-fixed, paraffin-embedded tissue blocks. After de-paraffinisation, the sections were submitted to heat-induced epitope retrieval by boiling for 20 min in 1 mmol/L sodium citrate buffer (pH 8.0). Primary antibodies used were antibodies against alpha-sarcomeric actinin (mouse IgM, 1:400, Sigma), c-kit (rabbit, 1:100, DAKO), CD 45 (mouse IgG, 1:100, DAKO or rat, 1 µg/ml, Abcam), mast cell tryptase (mouse IgG, 1:1000, Thermo scientific) and CD 68 (mouse IgG, 1:50 (DAKO). Nuclei were stained with DAPI (DAPI Mounting Medium, Vector Laboratories).

Confocal microscopy Nikon C1 system and Zeiss LSM510 system was used to establish the stainings and in selected stainings in the follow up. ZEISS Observer Z1 was used to quantify the cells after establishing the stainings.

Statistic was performed with SPSS (Version 19). C-kit+ cells are given as percentage of nuclei of c-kit+ cells/all nuclei counted. Values are given as mean ± SD. In the case of multivariate analysis, a one way anova was performed. As post hoc analysis a Dunnett T3 was used. Two groups were tested with Mann–Whitney test. p values $p < 0.05$ were considered significant.

Results

To evaluate a possible influence of pressure overload on the number of cardiac resident stem cells, eight specimens

from patients with univentricular right ventricular pressure overloaded hearts were investigated. Samples from explanted hearts from patients with dilated cardiomyopathy served as a control group. The age of the explanted hearts ranged from 16 days to 13 years. Right ventricular biopsies ($n = 14$) from heart-transplanted patients served as a second control group. The age of the patients at biopsy sampling ranged from 2 months to 19 years.

Immunostaining of cardiac specimens is shown in Fig. 1. The typical cardiomyocyte structure can be clearly visualized by staining with alpha-sarcomeric actinin. Some single c-kit + cells can be seen (Fig. 1c), while others are located in so-called stem cell niches (Figs. 1a–e, 2).

In biopsies of heart-transplanted patients (2 months–19 years), we observed 0.15 ± 0.16% c-kit+ cells. However, we did not see a significant correlation between the number of c-kit+ cells and the time span between birth and biopsy sampling (Fig. 3). We also did not find a significant correlation between the number of c-kit+ cells and the time span between birth and sampling at the time point of heart explantation in patients with dilated cardiomyopathy (data not shown).

Since previous experimental studies [10, 12], suggested that pressure overload might influence the number of c-kit+ cells in the right ventricle, we evaluated specimens derived from patients with pressure overloaded single right ventricles ($n = 8$) in comparison to specimen from patients after heart transplantation ($n = 14$) and to specimen from explanted hearts from patients with dilated cardiomyopathy ($n = 4$). The number of c-kit +cells from specimen from patients with pressure overloaded single right ventricle was significantly higher compared to specimen from patients after heart transplantation (0.84 ± 0.62% vs. 0.15 ± 0.16) and showed a borderline significance compared to specimens from patients with dilated cardiomyopathy, (0.84 ± 0.62% vs. 0.22 ± 0.22% (Fig. 4).

We also counted c-kit+ cells in explanted hearts as c-kit+ cells/field. In this case, the increase of c-kit+ cells was 4.8 times higher in specimens from patients with pressure overloaded single right ventricles compared to patients with dilated cardiomyopathy ($p < 0.01$).

Recently, it was claimed that c-kit+ cells in fact are cardiac mast cells Zhou et al [35]. To evaluate the contribution of cardiac mast cells to the c-kit+ cells, and to rule out contamination of cells from the leucocyte lineage and from macrophages, specimen were further analysed for CD45, CD68 and mast cell tryptase. We could not detect any CD68 cell additionally expressing c-kit ($n = 3$);

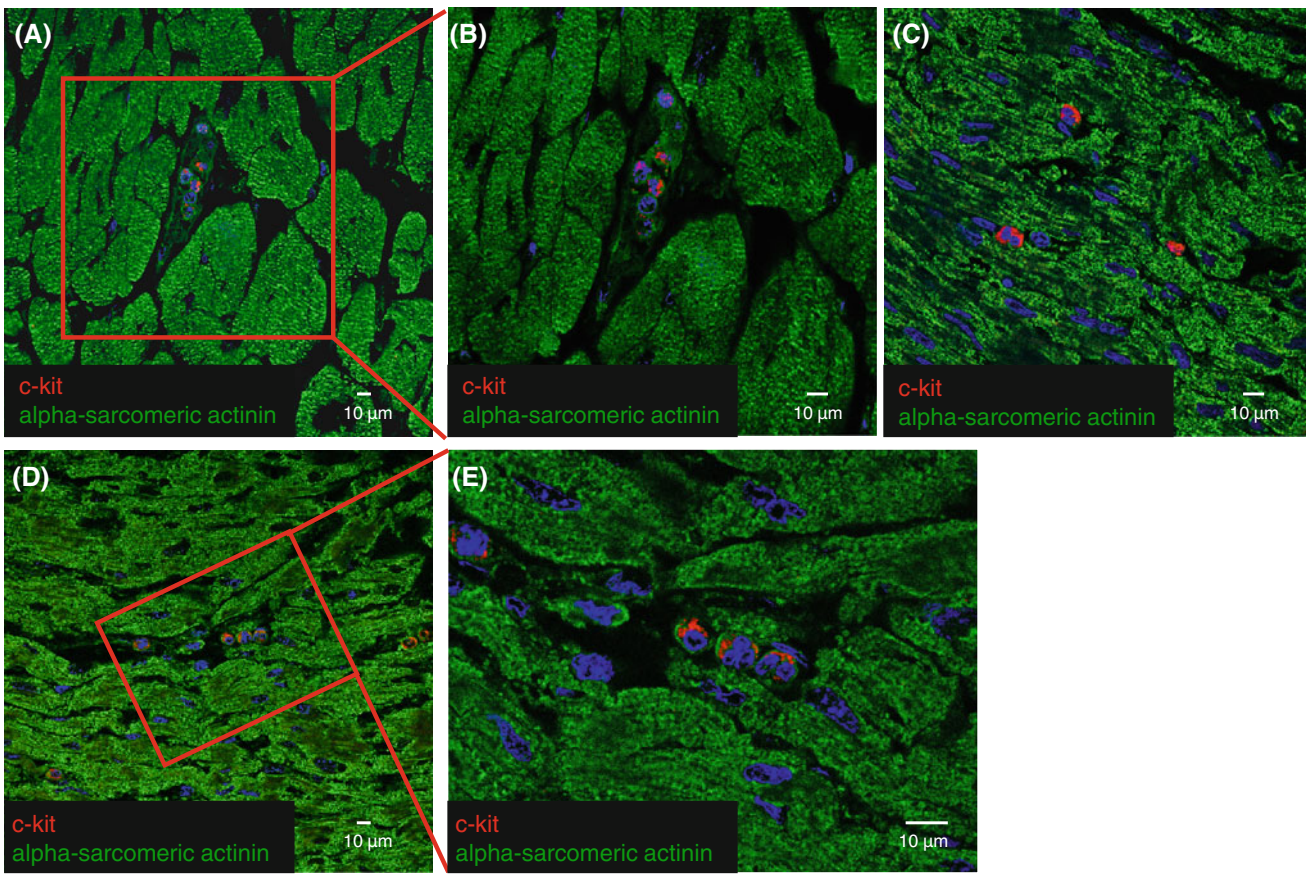

Fig. 1 **a–e** Alpha-sarcomeric actinin is stained in *green*, c-kit is stained in *red*. Nuclei are *blue* (DAPI). Some c-kit+ cells form clusters

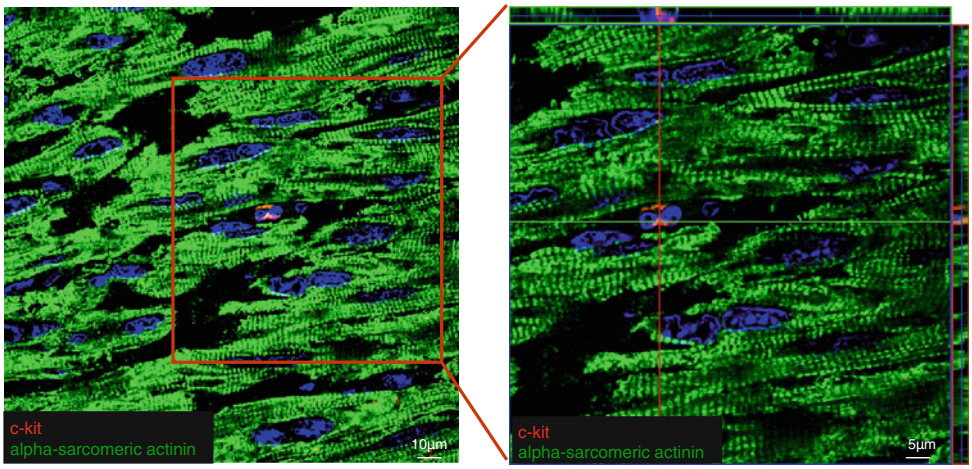

Fig. 2 z-Stack image of c-kit+ cardiac cells. Alpha-sarcomeric actinin is stained in *green*, c-kit is stained in *red*. Nuclei are *blue* (DAPI)

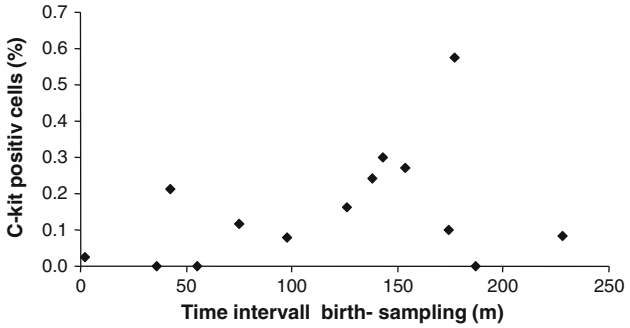

Fig. 3 The number of c-kit+ cells in each biopsy is shown. The age of the patients at biopsy sampling ranged from 2 months to 19 years. There is no time dependency between the number of c-kit+ cardiac cells and the time point of biopsy sampling in patients after heart transplantation

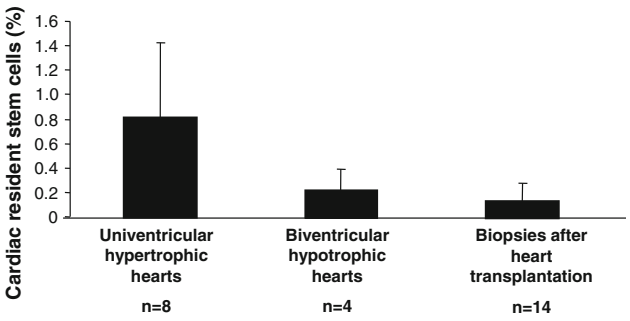

Fig. 4 The number of c-kit+ cardiac cells is shown in patients with univentricular hearts from the right ventricular type and pressure overloaded hypertrophic conditions in the first line. The number of c-kit+ cells from patients with biventricular hearts with dilated cardiomyopathy are shown in line two, the c-kit+ cells from specimen from patients after heart transplantation are shown in line three. A one way anova was performed (p for trend = 0.0048). In the Dunnett T3 post hoc analysis a borderline significance was found for column 1 versus 2, a significant difference was found between column 1 versus 3

therefore, we focused on a co-staining of CD45 and mast cell tryptase, and again specimen from the patients with pressure overloaded single right ventricles and specimen

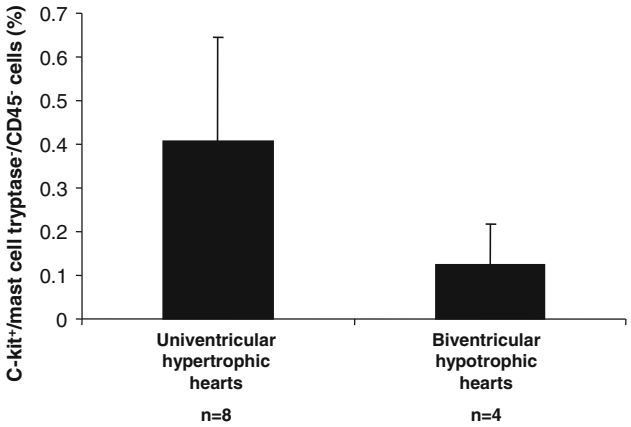

Fig. 5 The number of c-kit+/CD45-/mast cell tryptase- cardiac resident cells is shown in patients with univentricular hearts from the right ventricular type and pressure overloaded hypertrophic conditions in the first line and from patients with biventricular hearts with dilated cardiomyopathy in line two. There was a significant difference ($p < 0.05$; Mann–Whitney)

from patients with dilated cardiomyopathy were analysed. Under conditions of pressure overload, the right ventricle shows a significant increase in c-kit+/mast cell tryptase-/CD45- cells compared to the right ventricle of patients with dilated cardiomyopathy (0.41 ± 0.24 vs. 0.13 ± 0.09; $p < 0.05$; Figs. 5, 6). These data confirm that the increase in c-kit+ cell is not caused by an accumulation of hematopoietic cells, macrophages or mast cells that co-express c-kit.

Discussion

In the present study, we demonstrate that the number of cardiac resident stem cells, defined as c-kit+/mast cell tryptase-/CD45- negative cells, is significantly augmented in human hearts exposed to pressure overload. The number of c-kit+/mast cell tryptase-/CD45- cells was approximately

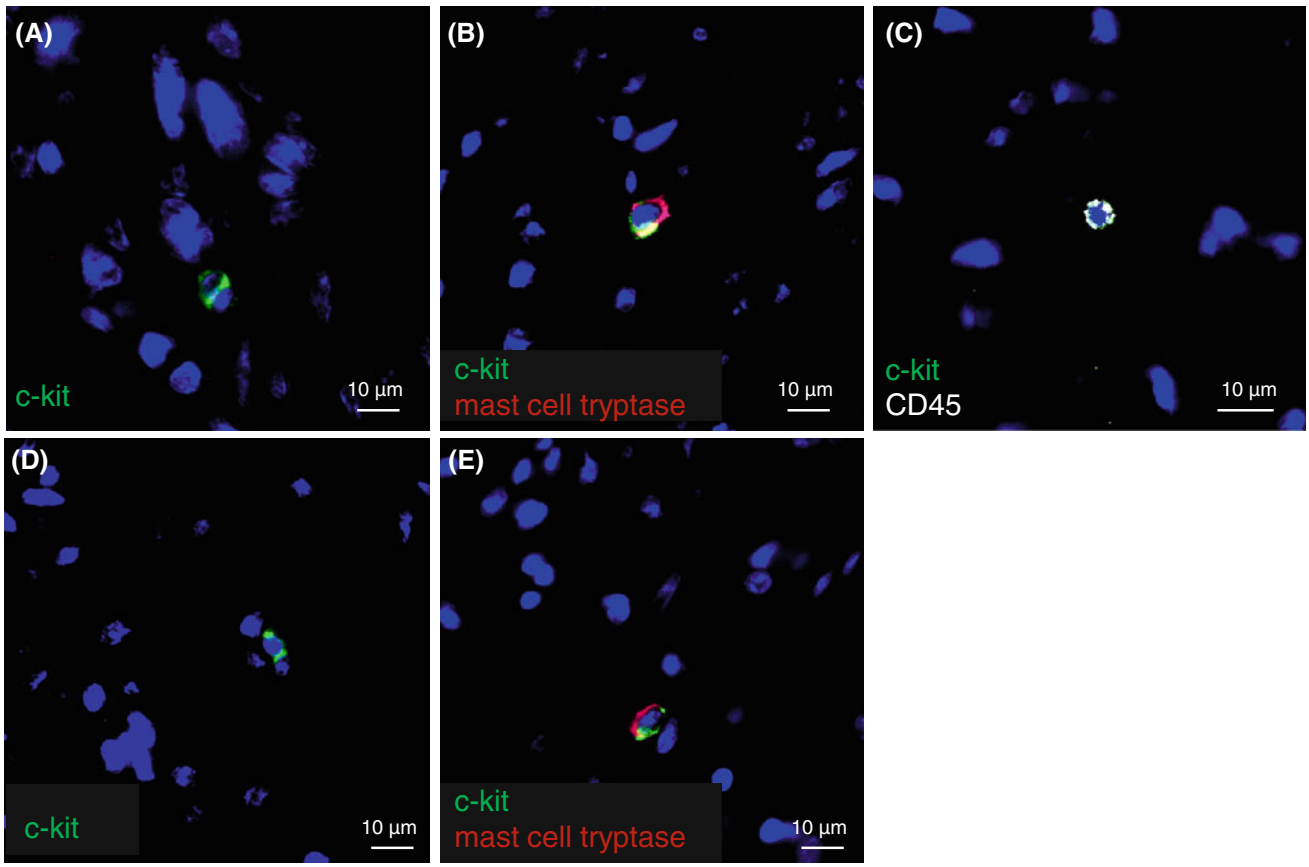

Fig. 6 Expression of mast cell and hematopoietic markers in c-kit+ cells. All specimen are co-stained with antibodies against c-kit, mast cell tryptase and CD45. C-kit is indicated by *green* , mast cell tryptase by *red*, CD45 by *white*, Nuclei are *blue* (DAPI). Specimen from univentricular hearts with pressure overload are shown in the *top panel*, specimen from patients with biventricular hearts with dilated cardiomyopathy are shown in the *lower panel*. **a**, **d** C-kit +/mast cell tryptase-/CD45- cells are depicted. **b**, **e** C-kit +/mast cell tryptase +/CD45- cells are depicted. **c** A c-kit +/mast cell tryptase -/CD45+ cell is depicted

three-fold higher in heart specimens of patients with chronic pressure overload compared to patients with dilated cardiomyopathy or to patients after heart transplantation. This increase in c-kit+ cell is not caused by an accumulation of hematopoietic cells, macrophages or mast cells that co-express c-kit. Up to now, different studies showed decreasing numbers of cardiac resident stem cells with increasing age of children/patients [1, 23]. The number of c-kit+ cells in the right ventricle declines to around 0.1% in the first months of life [1]. The number of c-kit+ cells were not down-regulated in the present study which investigated heart tissue obtained from heart-transplanted patients from 2 months to 19 years.

A previous study described a four-fold increase of c-kit+ cells in patients with heart failure in the free wall of the left ventricle [14]. However, these results were derived from a perfusion based cell isolation protocol. Moreover, these cells frequently co-expressed c-kit and CD45 suggesting a leukocyte origin of the cells. In the present study, we also detected about 7% of the c-kit+ cells being positive for CD45. We restricted the inclusion criteria to explanted pressure overloaded single right ventricle with the absence of being on a ventricular assist device or the diagnoses of having a sepsis and extended the patients age and excluded cardiac mast cells/CD45+ cells and CD68+ cells. Using these stringent criteria for counting c-kit+ cells, we identified an approximately three-fold increase in c-kit+ cells in pressure overloaded right ventricular hearts.

In comparison a 8 to 19-fold increase of c-kit+ cells has been described under the condition of aortic stenosis in the left ventricular outflow tract, supporting the theory that pressure overload leads to an increase of cardiac resident stem cells [34]. However, the absolute number of c-kit+ cells was extremely low (approximately 10 c-kit+ cells/cm^2) and the highest number of c-kit +cells was found in the apex with significantly lower numbers in other parts of the ventricle [15]. In addition, a 1.5-fold increase of c-kit+ cells was found in specimen from patients with hypoplastic left heart syndrome (mean age of 113 ± 125 days) compared to control individuals [6].

Although we carefully evaluated the co-expression of hematopoietic and mast cell markers in c-kit+ cells in the

present study, we cannot formally prove that the detected cells are indeed functional cardiac stem cells. Indeed, recently c-kit+ cells were observed in the heart, which had an bone marrow-derived origin [7], or co-expressed markers for the endothelial lineage [31]. Furthermore, non-stem cells of cardiac origin [33] or secretome of peripheral blood cells [19] have been shown to initiate c-kit expression in differentiated cells and c-kit expression is required for cardiomyocyte terminal differentiation [17], migration [13] and regeneration [21, 25].

Taken together, our data provide circumstantial evidence that pressure overload induces an increase in c-kit+/mast cell tryptase-/CD45- cells in the heart suggesting an activation of the cardiac resident stem cell pool. Cardiac malformations constitute of a variety of different underlying diseases. The situation gets even more complicated and the potential patients' specimen get more heterogeneous by the fact that for example a single ventricle pathophysiology is in most cases operated and palliated in three steps leading to different hemodynamics and changing pressure and volume conditions over time. We are, therefore, aware that, despite the fact that a pressure overload condition was present in all cases, our patient cohort is heterogeneous.

It is obvious that in humans the intrinsic cardiac regenerative capacity is insufficient to restore contractile function after substantial cardiac injury. Understanding the mechanism leading to an increase of cardiac resident stem cells in conditions of pressure overload might open novel possibilities to augment cardiac stem cells also in other patient groups with congestive heart failure and to adopt new therapies based on this concept of augmentation of cardiac resident stem cells.

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