Characterization of long-term endogenous cardiac repair in children after heart transplantation

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Aims
Circulating cells repopulate the heart at a very low rate in adult humans. The knowledge about time-dependent cardiac regeneration is very limited and the contribution of circulating cells to cardiomyocytes or vascular cells in children is unknown. This study investigates the endogenous repair capacity and the long-term incorporation of circulating cells in heart-transplanted children.

Methods and results
Cardiac and endothelial chimerism was detected in endomyocardial biopsies of nine children (age 1 months–14 years) with sex-mismatched heart transplantation by fluorescence in situ hybridization. Time from transplantation to biopsy ranged from 1 month up to 10 years. The extent of repopulating cardiomyocytes was $2.39 \pm 1.54\%$ (range: 0–4.2%) and correlated significantly with the time from transplantation to biopsy sampling ($r^2 = 0.69, P = 0.006; n = 9$). The calculated contribution of male cardiomyocytes in the female heart per year was $0.36 \pm 0.09\%$. Consistent with the previous reports, the incorporation of vascular cells was higher compared with cardiomyocytes ($14.4 \pm 4.17\%$), but did not correlate in a time-dependent manner.

Conclusion
Circulating cells contribute to cardiomyocytes and endothelial cells in children after heart transplantation. The incidence of repopulating cardiomyocytes continuously increases in a time-dependent manner ($\sim 4\% \ Y\text{-}chromosome^+\ cardiomyocytes/10\ years$) and resembles the cardiac regeneration activity observed in adults.

Keywords
Heart failure • Cardiac regeneration • Cardiac chimerism • Progenitor cells

Introduction
The paradigm that the heart is a post-mitotic organ without regenerative potential was challenged during the last years. Typically, adult cardiomyocytes do not re-enter the cell cycle, when exposed to growth signals, and their predominant form of growth is increase in cell size (hypertrophy). However, the observation that cardiomyocyte apoptosis not only induces heart failure, but can also occur under physiological conditions ($0.001–0.002\%$ apoptotic cardiomyocytes$^1$) questioned the dogma of a post-mitotic organ. A new concept of cardiac homeostasis was proposed (for review, see Nadal-Ginard et al.$^{2,3}$). It has been suggested that cardiomyocyte loss (e.g. by apoptosis) is compensated by the regeneration of cardiomyocytes to maintain tissue homeostasis under physiological conditions. Indeed, a recent genetic fate-mapping study demonstrated that substantial cardiomyocyte renewal from extracardiac progenitor cells occurs after myocardial injury or pressure overload.$^3$ However, in chronic pathological conditions of cardiac decompensation, endogenous repair capacity may not be sufficient and new therapeutical options are needed to further enhance the cardiac regeneration.

Tissue-resident cardiac stem cells were identified, which proliferate and differentiate to cardiomyocytes upon injury.$^4$ However, not only endogenous cardiac resident stem cells, but
also extracardiac progenitors may contribute to the formation of new cardiomyocytes and tissue homeostasis. The first observation that circulating cells contribute to cardiac repair in adult humans was published in 2002 by Quaini et al., who identified male cardiomyocytes in female hearts after heart transplantation. Cardiac chimerism was reported to achieve values as high as 9% (calculated cardiac chimerism 18%). Subsequent studies showed varying numbers of cardiac chimerism ranging from 0.04 to 6%.5–11 One study did not detect cardiac chimerism at all.12 Of note, all these studies have been performed in adults and the time between transplantation and assessment of cardiac chimerism was limited.  

Ageing and cardiovascular risk factors are well known to limit stem and progenitor cell function.13–15 Children typically lack adult cardiovascular risk factors, such as acquired diabetes, hypercholesterolaemia, hypertension, smoking, or other risk factors, and might represent a population with a higher endogenous repair capacity.  

Therefore, we investigated the endogenous repair capacity in children to assess whether the incidence of cardiac repopulation might be enhanced during this juvenile state. Moreover, the long-term contribution of endogenous circulating cells to the replacement of cardiomyocytes was determined in children up to 10 years after heart transplantation.  

Methods

Specimen selection

Nine biopsies from gender-mismatched heart-transplanted children (female donor–male receptor) were studied. Rejection was evaluated histologically in these biopsies and only biopsies with rejection grade ISHLT 0 or 1A were evaluated for time-dependency of cardiac chimerism. Patients’ characteristics including the underlying heart disease, patient age, donor age, and time of biopsy are summarized in Table 1.  

In addition, three biopsies with acute rejection (ISHLT 3A or 4) in the last 30 days before biopsy sampling were analysed for cardiac chimerism. Two gender-matched biopsies from male and female patients served as controls.

Endomyocardial biopsies were taken according to the post-transplantation evaluation programme in the pediatric heart transplantation centre 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.

Fluorescence in situ hybridization

According to the standard procedures, 6 μm sections were prepared from formalin-fixed, paraffin-embedded tissue blocks. After deparaffinization, sections were submitted to heat-induced epitope retrieval by boiling for 22 min in 1 mmol/L sodium citrate buffer (pH 8.0). Sections were fixed with 1% paraformaldehyde/PBS on ice for 10 min.

The X and Y-chromosomes were detected by fluorescence in situ hybridization (FISH) with ALPHA satellite probes from VISIS (labelled with spectrum green and spectrum orange, respectively). Final concentration used was 10.5 μL hybridization buffer, 1 μL distilled water, 1 μL X probe, and 1 μL Y probe. Probes were dehybridized for 4 min at 71°C before incubation of the probes with the sections (4 min at 80°C). Overnight hybridization at 37°C was followed by a stringent wash (2× SSC with 50% formamide two times for 10 min, 2× SSC for 5 min, NP-40/2× SSC for 5 min, 2× SSC for 5 min at 42°C).

Sections were blocked for 30 min with bovine serum albumin and incubated with antibodies against α-sarcosomic actinin (mouse IgG, 1:400, Sigma), Troponin I (rabbit, 1:100, Santa Cruz) or CD31 (mouse, 1:25, DAKO) for 1 h. After washing, cells were incubated with species-specific Alexa Fluor 647-conjugated secondary antibodies (1:200) (donkey anti-mouse IgG, Dianova; goat anti-rabbit, Molecular Probes; donkey anti-mouse IgG, Molecular Probes, respectively). Nuclei were stained with DAPI (DAPI Mounting Medium, Vector Laboratories) or Sytox blue (1:2000, Molecular Probes). Confocal microscopy (Zeiss LSM510 system, Germany) was used to analyse the stained biopsies.

Statistic

Values are given as percentage mean ± SEM. Correlation between variables was tested with Pearson’s correlation coefficient. P-values <0.05 were considered significant.

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HTx, heart transplantation; lymphoc, lymphocytes; BNP, brain natriuretic peptide; ISHLT, International Society for Heart and Lung Transplantation; CHD, congenital heart disease; CMP, cardiomyopathy.
**Results**

The hybridization efficiency and the specificity of the FISH technique was determined in heart biopsies from non-mismatched transplanted female and male children serving as negative and positive controls, respectively. No Y-chromosome was detected in the control female heart, indicating a high specificity of the staining, as shown in Figure 1A. In the case of male gender-matched positive control, 79.3 ± 4.4% of cardiomyocytes were positive for the Y-chromosomes. The number of X-chromosomes in the control male hearts was 73.3 ± 6.5%. A representative image for male positive control is provided in Figure 1B. Although the FISH protocol partially impaired the quality of the troponin I or α-sarcomeric staining (compare Figure 1C without FISH and Figure 1D-F with FISH), the α-sarcomeric actinin staining was sufficient to identify X and Y-chromosome-positive cells in the heart biopsies (Figure 1D). Cells that fulfilled the criteria to be counted as cardiomyocytes are shown in Figure 1E and F. These cells display Y-chromosomes located in the nucleus and were surrounded by α-sarcomeric actinin (Figure 1E and F).

To assess the cardiomyocyte chimerism in mismatched heart transplantation, the database of the Pediatric Heart Transplantation centre in Giessen was screened. Nine male children (age 1 month–14 years) were identified, who had received a female cardiac allograft and in whom rejection was ruled out by an independent pathologist. All biopsies analysed were grouped in Grades 0–1A according to the rejection criteria of the International Society of Heart and Lung Transplantation (ISHLT). The number of Y-chromosome cells in the biopsies from the right ventricle was 2.39 ± 1.54% varying from 0 to 4.2% with the lowest value at the shortest time after transplantation (1 month) and the highest value 10 years after transplantation (Figure 2). The number of α-sarcomeric actinin cells correlated significantly ($P = 0.006; n = 9$) with the time span between heart transplantation and biopsy sampling ($r^2 = 0.69$, Figure 2). The contribution of male cardiomyocytes to the female heart approximates $0.36 \pm 0.09\%$ per year. These data were confirmed by troponin I staining ($n = 6$). The number of Y-chromosome cells expressing troponin I was $2.5 \pm 1.46\%$ (range 0.6–4.4%). Similar to what has been shown for α-sarcomeric actinin staining, the number of Y-chromosome troponin I cells correlated with the time span between heart transplantation and the time point of biopsy sampling ($r^2 = 0.98, P < 0.005$, Figure 2).

To determine the effect of acute rejection, cardiac chimerism was also detected in three biopsies derived from sex-mismatched

**Figure 1** Immunostaining of heart biopsies. (A) A female gender-matched heart biopsy stained for X- and Y-chromosomes displays only white-stained X-chromosomes (marked by an arrow). Nuclei (blue) are stained with Sytox blue. (B) A male gender-matched biopsy is shown. X- and Y-chromosomes are stained and marked by an arrow. White dots indicate X-chromosomes (section symbol), green dots Y-chromosomes (asterisk). Nuclei (blue) are stained with Sytox blue. (C) Sarcomeric structures are visualized by staining with antibodies against α-sarcomeric actinin (red). Nuclei (blue) are stained with Sytox blue. (D–F) Male (receptor)–female (donor) gender-mismatched biopsies. After fluorescence in situ hybridization, actinin staining still can be used to detect the cardiomyocytes (red). Nuclei (blue) are stained with Sytox blue. (D) Y-chromosome staining is indicated in green (asterisk), X-chromosome staining is indicated in white. Two cells marked by an arrow are positive for Y-chromosomes. These α-sarcomeric actinin cells do not represent cardiomyocytes. (E) Y-chromosome staining is indicated in green. Two male cells are marked by an arrow. The cell additionally marked with an asterisk represents a cardiomyocyte. F) A cardiomyocyte, indicated by the presence of an X-chromosome (white, marked by a section symbol) and Y-chromosome (green, marked by asterisk) located in the nucleus surrounded by sarcomeric structures is shown.
children (age 2 months–4.6 years), who experienced an acute rejection (ISHLT 3A or 4) during the last 30 days before biopsy sampling. These samples showed a higher degree of chimerism (3.85, 4.51, and 6.20%).

To assess the chimerism of vascular cells, six of nine biopsies were stained for CD31. The percentage of CD31+ and Y-chromosome+ cells out of all CD31+ cells varied between 9.3 and 20.0% with a mean of 14.4 + 4.17% (Figure 3) and was higher compared with the incidence of cardiomyocyte chimerism. Interestingly, the number of CD31+/Y-chromosome+ cells was already high at early time points after transplantation. However, the turnover of CD31+ cells in the long-term follow-up did not correlate well with the different time points of biopsy sampling ($r^2 = 0.1, P = 0.5$).

**Discussion**

The present study provides evidence that transplanted hearts are repopulated by extracardiac progenitors in a time-dependent manner in children. This study extends knowledge from previous studies assessing cardiac chimerism in adult patients with heart transplantation. The first study in 2002 reported occurrence of cardiac chimerism in up to 18% of cardiomyocytes. Following studies proposed lower values between 0 and 6% cardiomyocyte chimerism. The variable numbers might be explained by various factors, such as technical issues and the clinical status of the patients (patient history, e.g. rejection). As discussed by Anversa and coworkers, confocal microscopy represents the instrument of choice, where the thickness of optical sections can be chosen to minimize the false-negative or false-positive results. Another important point is to rule out acute rejection, because infiltrating cells can lead to false-positive results as discussed by Laflamme. These two major critics were taken into account in the present study in order to gain most valid data. The FISH protocol was optimized to yield a sensitivity of >70% for the detection of Y-chromosome.

The time-dependency of cardiac chimerism documented in the present study supports the concept that cardiomyocytes are continuously generated by extracardiac cells. However, the origin of the repopulating cells cannot be precisely identified in the present study. Although controversially discussed, several groups have demonstrated that bone-marrow-derived circulating cells can acquire a cardiomyogenic phenotype. Cardiac chimerism can be detected in sex-mismatched bone-marrow-transplanted patients. However, recent studies suggest that, at least under stress conditions, non-bone-marrow-derived progenitor cells are also mobilized in experimental models. These circulating tissue- or vascular-wall-derived cells can home to sites of ischaemia, albeit their cardiac regeneration capacity has not been addressed so far. Finally, we cannot fully exclude that detected Y-chromosome+ cells derive from a small amount of atrial host myocardium typically retained after surgery.

Our initial hypothesis was that the extent of cardiac chimerism might be higher in children compared with adults. However, the extent of chimerism detected in the present study was still rather low and was within the range detected in other studies excluding rejection episodes. Although we might have missed some cardiomyocytes because of the rigorous criteria used for counting and the limited sensitivity of the FISH, it is unlikely...
that more than 1–2% of the cells are replaced per year in the present study.

These results are supported by a recent genetic fate-mapping study, demonstrating that substantial cardiomyocyte renewal requires prior myocardial injury or pressure overload. Thus, under unstrained condition lacking injurious insults, there appears to be no need for significant cardiac regeneration. Nevertheless, one has to bear in mind that this study was performed in heart-transplanted children. Constant immunosuppression to limit organ rejection (even if documented grade was ISHLT 0–1A) might lead to a different regeneration potential of cardiomyocytes in transplanted patients than seen under physiologically non-injured non-transplanted condition. Therefore, the extrapolation of the present data to the situation in healthy human individuals has to be viewed cautiously.

Endogenous cardiac regeneration might be sufficient to repopulate cardiomyocytes under physiological conditions or in conditions with limited apoptosis. However, higher degrees of apoptosis as seen in patients with heart failure might not be adequately compensated by endogenous regeneration activity. Therefore, new therapeutic options are needed to enhance cardiac repair capacity. Since endogenous cardiac and extracardiac cells have the capacity to acquire a cardiac phenotype, cell therapy with autologous cells may be a useful tool. Indeed, several recent clinical trials suggest that bone-marrow-derived cells improve left ventricular ejection fraction and/or reduce infarct size. The improvement detected in clinical trials may indeed be in part caused by direct cardiac regeneration as indicated by the experimental studies. Furthermore, infused cells may mediate the effects on the endogenous regeneration capacity.

Our study additionally addressed to what extent the extracardiac cells contribute to vascular repair. We demonstrated that the number of vascular chimerism was greater compared with cardiomyocyte chimerism and did not show time-dependency, a finding which is consistent with the previous data from Minami et al. This might be explained by the fact that rejection starts immediately after transplantation, and activation and injury of the vascular wall therefore might enhance the early endothelial cell turnover.

In summary, the present results indicate that cardiac chimerism in children is time-dependent with a regeneration capacity of ~4% newly regenerated cardiomyocytes 10 years after transplantation. The present study supports the concept that cardiomyocytes are continuously regenerated during lifespan and might contribute to the maintenance of organ homeostasis.

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References


