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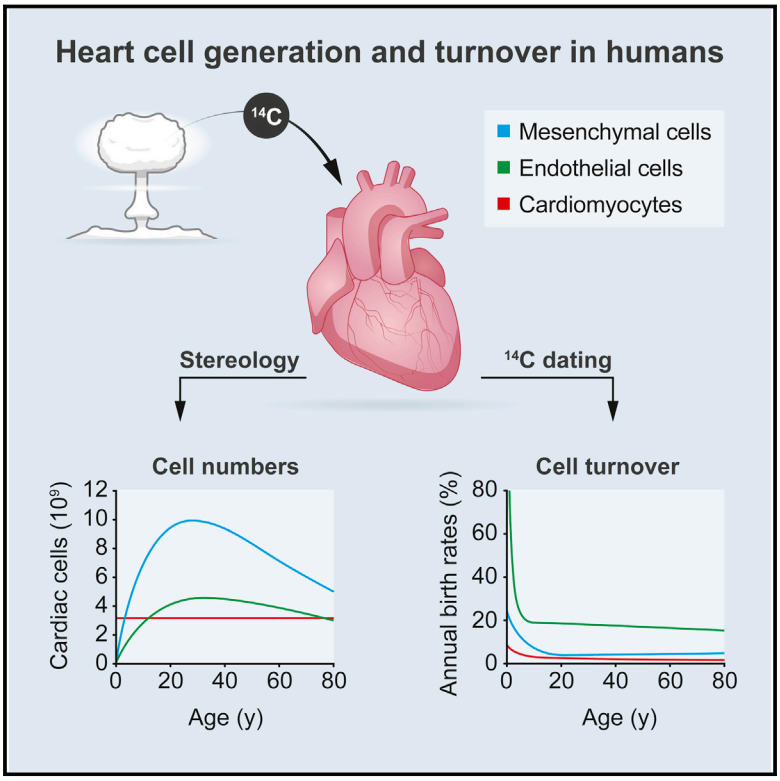
Dynamics of Cell Generation and Turnover in the Human Heart

Graphical Abstract



Highlights

- The number of cardiomyocytes remains constant during the human lifespan
- Endothelial and mesenchymal cells increase into adulthood and show high turnover
- Cardiomyocyte turnover decreases exponentially with age and is <1% per year in adults
- The cardiomyocyte turnover rate is equal in the main subdivisions of the human heart

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In Brief

A comprehensive analysis of cell generation and turnover in the human heart demonstrates that cardiomyocyte numbers are constant throughout the human lifespan, with a low turnover rate. Endothelial and mesenchymal cells are exchanged at a high rate, and their numbers increase into adulthood.

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Dynamics of Cell Generation and Turnover in the Human Heart

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SUMMARY

The contribution of cell generation to physiological heart growth and maintenance in humans has been difficult to establish and has remained controversial. We report that the full complement of cardiomyocytes is established perinataly and remains stable over the human lifespan, whereas the numbers of both endothelial and mesenchymal cells increase substantially from birth to early adulthood. Analysis of the integration of nuclear bomb test-derived ^{14}C revealed a high turnover rate of endothelial cells throughout life (>15% per year) and more limited renewal of mesenchymal cells (<4% per year in adulthood). Cardiomyocyte exchange is highest in early childhood and decreases gradually throughout life to <1% per year in adulthood, with similar turnover rates in the major subdivisions of the myocardium. We provide an integrated model of cell generation and turnover in the human heart.

INTRODUCTION

Loss of cardiomyocytes after a cardiac infarction or in heart failure is largely irreversible and constitutes a major health burden. Promotion of an endogenous regenerative capacity is an attractive concept for cell replacement. However, conflicting data regarding both the origin of new cardiomyocytes (Bersell et al., 2009; Ellison et al., 2013; Hsieh et al., 2007; Senyo et al., 2013; Uchida et al., 2013; van Berlo et al., 2014) and the extent of cell generation (Bergmann et al., 2009; Hosoda et al., 2009; Kajstura et al., 2010a, 2010b; Malliaras et al., 2013; Mollova et al., 2013) have made it difficult to assess if this is a rational and realistic prospect. The extent of cell generation in the human heart is one of the key questions in regenerative medicine and probably the one most difficult on which to reach a consensus.

Studies in mice have demonstrated that there is a dramatic drop in the generation of new cardiomyocytes the first few days after birth (Soonpaa et al., 1996; Walsh et al., 2010). A recent study suggested that an exception to this is a short burst of cardiomyocyte generation in juvenile mice, resulting in a doubling in the number of cardiomyocytes (Naqvi et al., 2014). In humans, a 3.4-fold increase in the number of cardiomyocytes from 1 to 20 years of age was recently reported (Mollova et al., 2013), whereas the development of the number of cells of the other main cell lineages in the myocardium has not been described.

The extent of cardiomyocyte exchange in the adult heart has been much debated, with estimates ranging between no turnover to all cardiomyocytes being exchanged every few years (for review, see Bergmann and Jovinge, 2014). Reports of relatively high numbers of cardiomyocytes with either apoptotic or mitotic markers in the adult human heart led to the conclusion that there must be substantial turnover of these cells (Beltrami et al., 2001; Kajstura et al., 2010a), but other studies have failed to reproduce high frequencies of potentially dividing cells (Mollova et al., 2013). Regardless, any estimate of cell turnover based

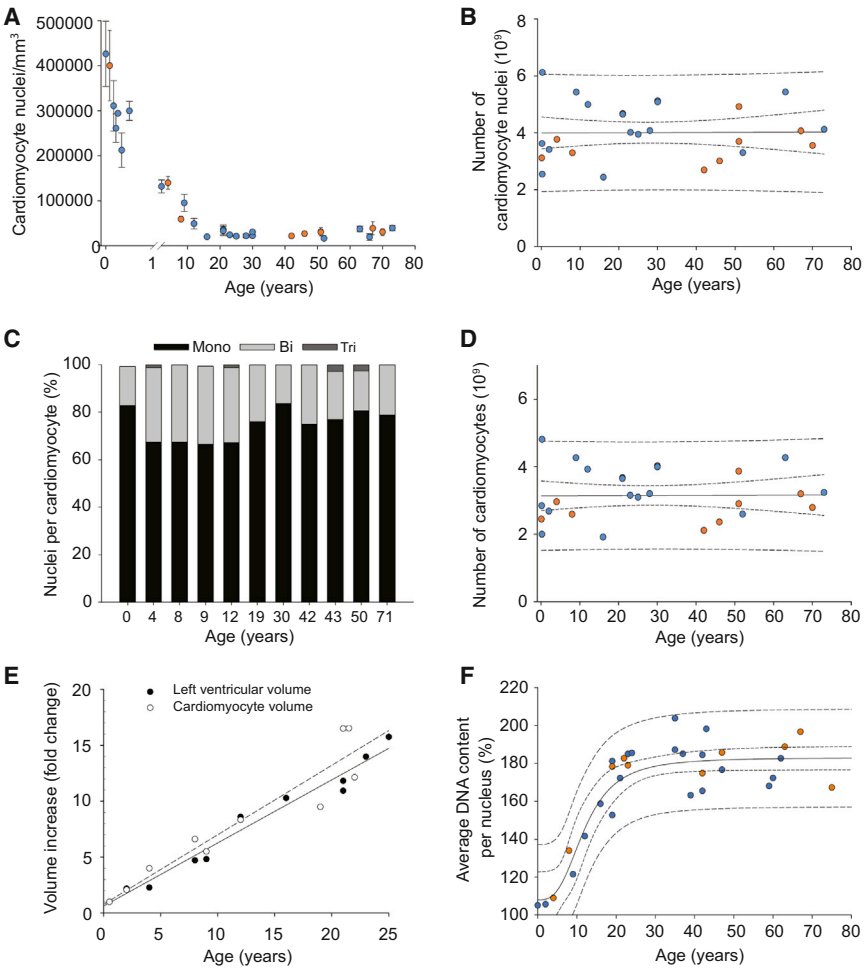

Figure 1. Cardiomyocyte Number, Volume, and DNA Content in Growing and Adult Hearts

(A) The density of cardiomyocyte nuclei declines in growing hearts and remains constant during aging.

(B) The number of cardiomyocyte nuclei in the left ventricle, calculated using the reference volume (see the Experimental Procedures), is stable postnatally.

(C) The ratio of mononucleated to multinucleated cardiomyocytes is already established at birth.

(D) Based on the number of cardiomyocyte nuclei and the level of multinucleation, the number of cardiomyocytes does not change significantly in growing hearts or with age ($R = 0.01$; $p = 0.96$).

(E) Comparison between left ventricular volume increase (black dots and black regression line) and average increase in cardiomyocyte volume (white dots and dashed regression line). Regression curves do not show any significant difference between the groups ($p = 0.67$; ANCOVA).

(F) Human cardiomyocyte nuclei are mostly diploid at birth and start to ploidize mainly in the second decade of life (100% corresponds to diploid nuclei; 200% corresponds to tetraploid nuclei). Red data points indicate females, and blue data points indicate male subjects. Dashed lines indicate the prediction interval (long dashes) and the confidence interval (short dashes).

See also Figure S1.

on the presence of markers associated with cell division or death rests on assumptions of, for example, the length of the cell cycle or the apoptotic process. Moreover, based on marker expression, it is not possible to deduce whether a cell indeed will go on to divide or die.

Cell generation is studied in experimental animals by prospectively labeling dividing cells with, for example, thymidine analogs, but it is challenging to use this strategy in humans. One study in cancer patients receiving the thymidine analog IdU as a radiosensitizer concluded that 22% of the cardiomyocytes are exchanged annually (Kajstura et al., 2010b). Another strategy for birth dating cells relies on analyzing incorporation of nuclear bomb test-derived ^{14}C into genomic DNA (Spalding et al., 2005), and this approach indicated a much lower turnover rate in humans, with the majority of cardiomyocytes never being exchanged even during a long life (Bergmann et al., 2009).

It is important to establish the magnitude of heart cell generation and renewal in the growing and adult heart, not least when considering the development of therapeutic strategies to promote regeneration. One reason why it has been difficult to assess cell numbers and exchange rate in the heart is that there are several processes occurring in parallel, such as multinucleation, polyploidization, and cell volume increase, all of which may influence the analyses in different ways. We have analyzed all of these parameters and have birth dated cells by analyzing genomic ^{14}C concentration, and we provide an integrated model of heart cell generation and turnover in humans.

RESULTS

A Constant Number of Cardiomyocytes in Humans

We determined the number of cardiomyocytes by stereology in the postmortem heart from 29 subjects, without any history or sign of heart pathology, aged one month to 73 years (see the Experimental Procedures; Figure S1A). It is challenging to delineate individual cardiomyocytes in tissue sections (Ang et al., 2010), especially in the perinatal period when the cell density is very high. We therefore used antibodies to the cardiomyocyte nuclear marker pericentriolar material 1 (PCM-1) to circumvent this problem (Bergmann et al., 2011). We obtained the reference volume of the left ventricle, either by direct measurements ($n = 12$) or by estimation based on echocardiography and MRI data ($n = 17$) (Experimental Procedures; Figures S1D and S1E). The density of myocyte nuclei decreased from 430,000 ± 72,000/mm^3 (mean ± SD) shortly after birth to 28,000 ± 7,200/mm^3 in subjects aged 20 to 73 (Figure 1A). The neonatal cardiomyocyte density reported in this study is in good agreement with studies by Mayhew et al. (409,000/mm^3)

and by Mandarim-de-Lacerda et al. (1997), who reported a density of 574,000/mm^3 in the third trimester of pregnancy (Mayhew et al., 1997). The resultant total number of myocyte nuclei did not show any age- (Pearson's r = 0.01; p = 0.96) or gender-related (two-sample t test; p = 0.10) differences (Figure 1B).

We found that 73.6% ± 7.0% of all cardiomyocytes were mononucleated; 25.5% ± 7.4% were binucleated; and 1.0% ± 1.2% were trinucleated (Figure 1C), in agreement with previous studies (Mollova et al., 2013; Olivetti et al., 1996). This ratio did not change substantially during heart growth or aging. Taking multinucleation into account, we established that already 1 month after birth (youngest analyzed subject), the final number of cardiomyocytes was reached ($3.2 \times 10^9 \pm 0.75 \times 10^9$ cells) and remained constant over the lifetime (Pearson's r = 0.01, p = 0.96; Figure 1D). This was independently corroborated by birth-dating analysis (see below). This result is in stark contrast to the recently reported 3.4-fold increase in cardiomyocyte number between 1 and 20 years of age (Mollova et al., 2013). The identification of cardiomyocyte nuclei in tissue sections is challenging (Ang et al., 2010; Soonpaa and Field, 1998). Strategies relying only on cytoplasmic or membranous cardiomyocyte markers (Mollova et al., 2013) do not allow an unequivocally identification of cardiomyocyte nuclei (Figures S1B and S1C) and therefore might introduce a quantification bias. It is likely that our use of a myocyte-specific nuclear marker allows for accurate identification and quantification of cardiomyocytes.

In order to determine the contribution of the increase in cardiomyocyte volume to the growth of the human heart, we stereologically measured the average volume of cardiomyocytes at different ages (Figure S1F; Experimental Procedures). We found that the growth of the left ventricle could fully be explained by the volume increase of cardiomyocytes (ANCOVA; p = 0.71) (Figure 1E). This was based on the relative volume of the left ventricle and cardiomyocytes, respectively, with age (in relation to $LV_{month\ 0–6}$: 12.4 g ± 3.4 g SD and $CM_{month\ 0–6}$: 2,557 μm^3 ± 666 μm^3 SD), indicating a mainly hypertrophic growth of the human left ventricle with the full complement of cardiomyocytes being established in the perinatal period.

The Cardiomyocyte DNA Content Increases during the Second Decade of Life

Many cardiomyocyte nuclei undergo DNA synthesis to become polyploid in humans (Adler, 1991; Bergmann et al., 2009; Herget et al., 1997; Mollova et al., 2013). Also, this has, however, been somewhat controversial, with one recent study concluding that the vast majority of cardiomyocyte nuclei in humans remain diploid throughout life (Kajstura et al., 2010b). As DNA synthesis during polyploidization influences the interpretation of any cell proliferation analysis, it is important to establish the kinetics of this process. We revisited the DNA content of human cardiomyocyte nuclei at different ages by flow cytometry.

We found that almost all human cardiomyocyte nuclei are diploid during the first years of life (Figure 1F). However, mainly during the second decade of life, the average DNA content per nucleus increases approximately 1.7-fold in the left ventricle, and thereafter it remains constant over the lifetime (n = 29; Pearson's r = −0.09; p = 0.68; Figure 1F). The right ventricle displays a slightly lower ploidy increase, 1.6-fold, compared to the left ventricle (p < 0.001; two-sample t test), which reaches into the third decade of life and remains constant thereafter (n = 19; Pearson's r = 0.32; p = 0.29; Figure S1G). Our analysis revealed that polyploidization occurs slightly later and for a more extended period than previously suggested by histological analysis (Adler, 1991). We did not find any significant differences in the nuclear ploidy within the left ventricle, comparing the apical and basal part of the left ventricle (paired t test; p = 0.68) or comparing the myocardial layers toward the endocardium with the layers toward the epicardium (paired t test; p = 0.92) (Figure S1H).

The Number of Endothelial and Mesenchymal Cells in the Myocardium Increases into Adulthood in Humans

Next, we made a stereological quantification of the number of endothelial cells, identified by binding of the lectin Ulex Europaeus lectin I (UEA I) (Conrad-Lapostolle et al., 1996), and mesenchymal cells, identified by the absence of markers for cardiomyocytes and endothelial cells (PCM-1 and UEA I negative) (Figure S2A). Mesenchymal cells comprise cells residing in the interstitium of the heart, including fibroblasts, pericytes, and smooth muscle cells, which are labeled with neither cardiomyocyte nor endothelial cell markers (Figure S2B). Approximately 99% of the cells in the PCM-1- and UEA I-negative fraction can be labeled by a mesenchymal cell marker combination, with antibodies against PDGFR-beta, fibroblast-specific protein 1, and smooth muscle actin (Figure S2B). However, 2.8% of the cells within the PCM-1- and UEA I-negative fraction express the lymphocyte common antigen CD45 (Figure S2C), and some of those CD45-positive cells co-express a mesenchymal lineage marker (PDGFR-beta, fibroblast-specific protein 1, and/or smooth muscle actin) (Figure S2D). The measured fraction of CD45-positve cells is however not large enough to significantly impact the interpretation of the ^{14}C measurements of the mesenchymal cell compartment (mesenchymal ^{14}C increases in average by only 2.8 units). This is based on the fact that mesenchymal cells are relatively young and that CD45-positive cells are of contemporary ^{14}C.

In the postnatal period, the majority of cell nuclei in the human left ventricle are cardiomyocyte nuclei (66% ± 1.7%; mean ± SD). During heart growth, the number of cardiomyocyte nuclei and cardiomyocytes remains constant, while the number of endothelial and mesenchymal cells expands 6.5- and 8.2-fold, respectively (Figures 2A and 2B), leading to an average of 18% ± 3.0% cardiomyocytes, 24% ± 4.7% endothelial cells, and 58% ± 5.6% mesenchymal cells in young adults. During aging, the endothelial and mesenchymal cell counts show declining trends (Pearson's r = −0.58; p = 0.10 and r = −0.56; p = 0.09, respectively; Figures 2A and 2B).

Isolation and Carbon Dating of Cell Nuclei from the Human Heart

Cell nuclei were isolated from human postmortem hearts and incubated with antibodies against PCM-1 and UEA I. Cardiomyocyte (PCM-1-positive), endothelial (UEA I-positive), and mesenchymal (PCM-1- and UEA I-negative) cell nuclei were isolated by flow cytometry (Figure 3; Figure S3; the Experimental Procedures) (Bergmann and Jovinge, 2012; Bergmann et al., 2011). UEA I binds specifically to human endothelial cells,

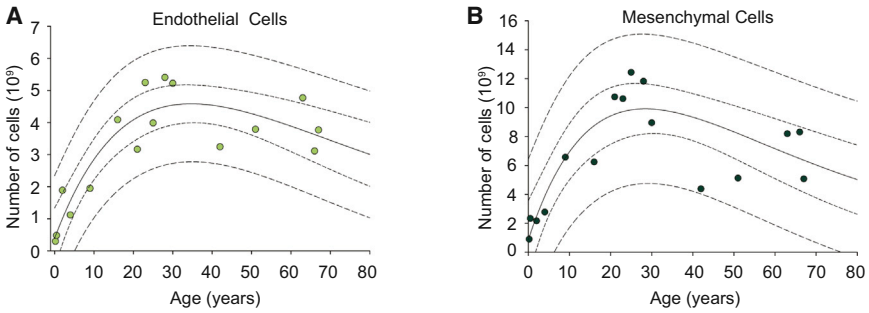

Figure 2. Postnatal Expansion of the Endothelial and Mesenchymal Cell Populations

(A and B) The number of endothelial and mesenchymal cells increases in growing hearts and declines modestly thereafter. Dashed lines indicate the prediction interval (long dashes) and the confidence interval (short dashes).

See also Figure S2.

and we found that UEA I not only binds to the endothelial cell membrane but also binds to the nucleus of cardiac endothelial cells (Figures 3B–3F). The adult human ventricles comprise 33% ± 15% (mean ± SD) cardiomyocyte nuclei, 24% ± 9.5% endothelial nuclei, and 43% ± 10% mesenchymal nuclei, proportions similar to those found in the stereological analysis (see above). The isolated nuclear populations were reanalyzed, and the purity of cardiomyocyte (96% ± 2.0%), endothelial (94% ± 5.4%), and mesenchymal nuclei (97% ± 5.7%) was determined (Figures S3A–S3C). Genomic DNA was isolated from the different cell populations, and the ^{14}C concentration was measured by accelerator mass spectrometry. ^{14}C concentrations were compensated for flow cytometry isolation impurities in the cell populations as described in the Experimental Procedures.

It is important to consider whether our isolated nuclei accurately reflect the cell populations in the heart or if there is an isolation bias for subtypes with different turnover kinetics. To assess that, we carbon dated not only genomic DNA from the isolated cell populations but also genomic DNA from non-sorted cardiac cell nuclei, as well as genomic DNA extracted directly from the myocardium. We calculated weighted ^{14}C averages of individual sorted populations (cardiomyocytes, endothelial, and mesenchymal cells) and compared those calculated ^{14}C values to the two other measurements. We did not find any significant differences in either of the approaches ($p = 0.31$ and $p = 0.76$, respectively, paired t test), indicating that ^{14}C-dated cardiac populations accurately represent the actual distribution in the heart (Figure S3D). Moreover, we verified that isolating cardiomyocyte nuclei based on cardiac troponins (^{14}C data from Bergmann et al., 2009) or PCM-1 labeling (Bergmann et al., 2011) has no impact on the obtained genomic ^{14}C concentrations (median test $p > 0.05$; power 90%; Figure S3E; see also Supplemental Experimental Procedures).

Generation and Exchange Dynamics of Endothelial and Mesenchymal Cells

We obtained ^{14}C data from subjects aged 20–74 years for endothelial ($n = 18$) and mesenchymal cell ($n = 26$) genomic DNA and related it to the atmospheric ^{14}C curve to assess cell generation and exchange (Figure 4A; Tables S1 and S2). The genomic ^{14}C concentrations of endothelial cells show levels rather close to contemporary levels and a low interindividual variability, indicating a high exchange rate of endothelial cells independent of age. Mesenchymal cell ^{14}C values are in general higher and show a bomb curve-dependent pattern, indicating slower turnover (Figures 4B and 4C).

To establish the cellular dynamics, mathematically modeled renewal rates based on data were obtained by retrospective ^{14}C dating and stereological data on cell numbers. Our core mathematical model describes cells defined by their age class, and while the individual grows older, the cells also grow older, making them move from age class to age class (transport equation). The solution to this equation is a distribution of cell ages, $n(t,a)$, which can be obtained for any chosen time point, for instance, the time of death of an individual. n in the equation below is cell density (the integral of n is cell number). t and a are subject age and cell age, respectively.

$$\frac{\partial n(t,a)}{\partial t}+\frac{\partial n(t,a)}{\partial a}=-\gamma n(t,a)$$

The solution is integrated with the bomb curve to yield the ^{14}C concentration in the cell population, which is modeled to be equal to the measured ^{14}C. Modeling consists of fitting the death rate of the cells and solving the birth rate that results in the measured cell number and ^{14}C. In the equation above, the death rate is represented by γ. The birth rate is formulated in a side condition, as an additional equation (equations are given in the Supplemental Experimental Procedures). The functional form of birth and death rates make up several versions of the described model; these versions are referred to as scenarios.

During adolescence and early adulthood, endothelial and mesenchymal cells go through a growth phase (phase A), followed by a slight decrease during adulthood (phase B) (Figures 2A and 2B). Consequently, we constructed a piecewise turnover model (phase A, B, and C) of several partial differential equations, allowing for more flexibility in fitting the cell number. In phases A and B the death rate was fitted and the birth rate adjusted to meet the change in cell number. We first evaluated a scenario with constant death rates after the growth phase for both endothelial and mesenchymal cells. The global fitting of the endothelial cells produced a very good fit, and the individual fittings showed no age-related correlation after the growth phase, indicating that the scenario was appropriate. The mesenchymal cells did not fit well with the same scenario and were instead modeled with an additional phase (phase C) spanning the years after middle age. The scenario that could reproduce the changing endothelial cell number predicted a

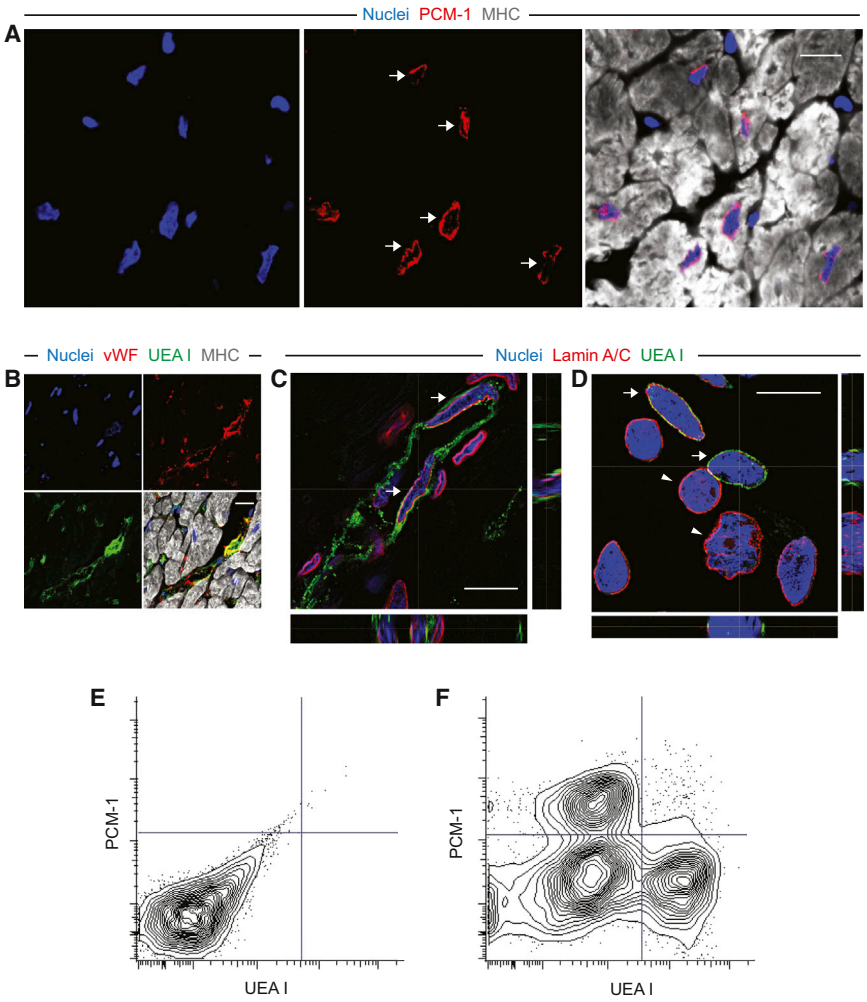

Figure 3. Identification and Isolation of Cardiomyocytes, Endothelial Cells, and Mesenchymal Cells

(A) Human cardiomyocytes were identified with antibodies against myosin heavy chain (gray). All myocyte nuclei, but not interstitial nuclei, show immunoreactivity for PCM-1 (arrows). Scale bars indicate 10 μm.

(B–D) The lectin UEA I binds specifically to human endothelial cells as shown and to their nuclei. The microscopy image of cardiac tissue sections (B) shows co-labeling of UEA I with the endothelial cell marker von Willebrand factor (vWF). Scale bars indicate 20 μm. Super-resolution images of cardiac tissue (C) and nuclear isolates (D) show binding of UEA I (arrows) to cardiac endothelial nuclei. The nuclear envelope is labeled with lamin A/C (red). Arrowheads indicate non-endothelial nuclei. Scale bars indicate 10 μm.

(E and F) Flow cytometry plots depict (E) labeling with isotype control antibodies and (F) co-labeling of cardiac nuclei with antibodies against PCM-1 and the lectin UEA I to isolate cardiomyocytes, endothelial cells, and mesenchymal cells.

See also Figure S3.

high birth rate of 123.0% per year at birth, declining to an approximately constant rate of 16.7% per year in adult life (Figure 4D). The mesenchymal cells showed lower rates with an estimated initial birth rate of 23.5% per year, declining to 3.9% per year in adult life (Figure 4D). The entire endothelial cell population will be exchanged approximately 9 times between 20 and 75 years of age (Figure 4E), whereas mesenchymal cells will be renewed around two times after the growth phase (Figure 4F).

Retrospective Birth Dating of Cardiomyocytes

The genomic ^{14}C concentrations in cardiomyocyte nuclei from 51 subjects, 8–75 years of age (Tables S1 and S2), were related to the atmospheric ^{14}C curve. There were no significant differences in genomic ^{14}C concentrations of myocytes residing in the myocardial layers toward the endocardium with the layers toward the epicardium of the left ventricle (paired t test; $p = 0.32$) (Figure 5A) or between the apical and basal part of the left ventricle (paired t test; $p = 0.80$) (Figure 5B). The ^{14}C data of all four locations showed a similar pattern, corresponding to ^{14}C levels within a few years after the birth of subjects born after the nuclear bomb spike, indicating a limited DNA turnover. Moreover, we wanted to establish whether the developmental and functional differences of the two ventricular systems were reflected in differences in cardiomyocyte renewal rates. Hence, we combined all measured ^{14}C values from the different left ventricular regions ($n = 32$) and compared them to genomic ^{14}C concentration obtained from right ventricular cardiomyocytes (Figure 5C). The genomic ^{14}C data from the right ventricle mirror the pattern of the left ventricular myocyte DNA, corresponding to ^{14}C levels within a few years after the birth of the subjects born after the bomb spike. Because cardiomyocytes undergo ploidization mainly in the second decade of life (Figure 1F), it is necessary to include the time course of ploidization in the mathematical model to infer renewal rates (see the Supplemental Experimental Procedures). To obtain a direct measure of cardiomyocyte turnover, independently of ploidization, we ^{14}C dated diploid cardiomyocyte nuclei separately (Figure S3F). Most data points corresponded to ^{14}C concentrations after the birth of the subjects, indicating postnatal myocyte renewal (Figure 5D).

Cardiomyocyte Age and Exchange Rate in the Human Heart

We applied the mathematical model described above for the analysis of ^{14}C data in cardiomyocytes. In agreement with the stereology data, the ^{14}C concentrations were not compatible with a substantial increase in cardiomyocyte cell number postnatally (see the Supplemental Experimental Procedures; ^{14}C simulation of cell number expansion during heart growth). The mathematically modeled scenario with a

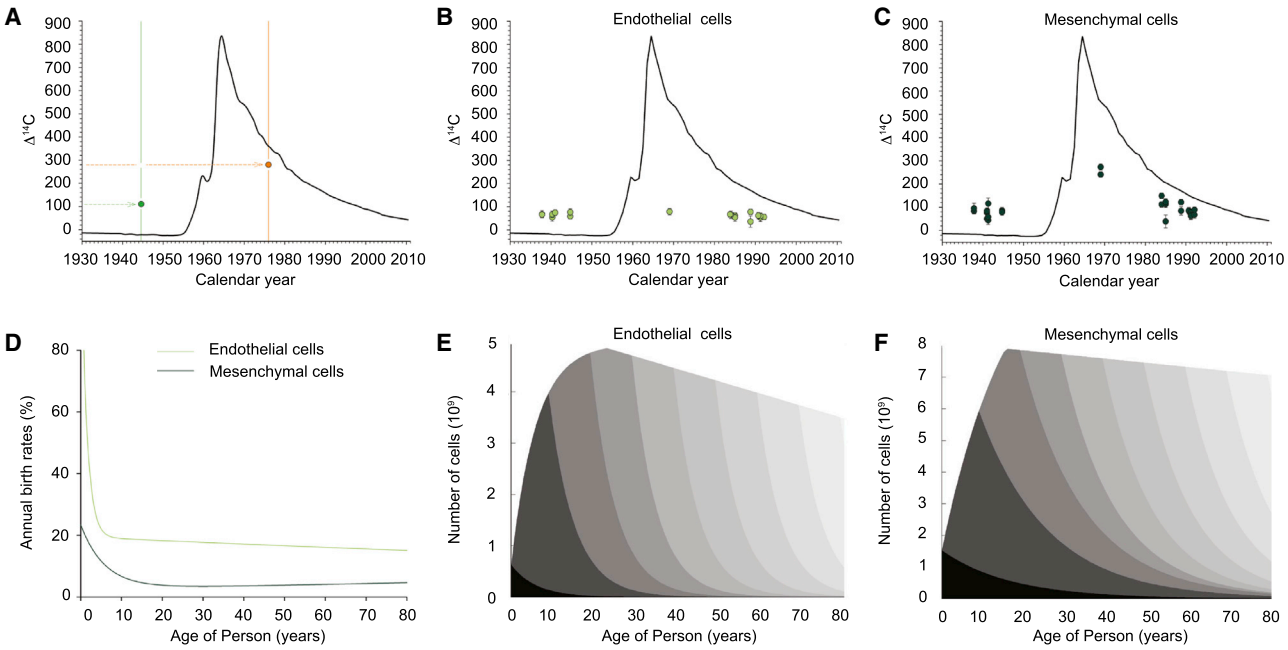

Figure 4. Renewal Dynamics of Non-cardiac Cell Populations in the Human Heart

(A) Schematic presentation of ^{14}C data. The black curve indicates the atmospheric ^{14}C concentrations over time. The colored dots represent the ^{14}C concentration in genomic DNA plotted on the date of birth (vertical lines) of the person before (green) and after (orange) the bomb spike. Genomic ^{14}C values corresponding to ^{14}C concentrations at the time of birth (data points on the atmospheric ^{14}C curve) indicate no postnatal and adult exchange of cells. Deviation from the ^{14}C curve indicates postnatal and adult genomic DNA turnover. The date of death of the subjects was 2002–2014 in all figures.

(B) Genomic ^{14}C concentrations of isolated endothelial cells are independent of the subjects' age and correspond to an endothelial cell age of approximately 5.8 years.

(C) Genomic ^{14}C concentrations of isolated mesenchymal cells indicate adult cell turnover with mesenchymal cells being on average 17.3 years.

(D) Annual birth rates of endothelial and mesenchymal cells decline in an exponential fashion in the growing heart. In the adult heart the birth rates of mesenchymal cells drop to values lower than 4%, whereas the endothelial birth rate remains almost constant at an annual rate of 17%.

(E and F) For a person's given age, postnatally generated cells are shown with different shades of gray, indicating the decade in which they were generated. Black indicates cells that were generated before birth; the darkest gray indicates cells generated during the first decade of life; and cells one shade lighter are generated between 10 to 20 years of age, and so on. (E) Accordingly, endothelial cells are rapidly replaced with more than 90% of all cells being replaced within one decade. (F) Mesenchymal cells show slower renewal with a cellular representation of almost all age classes in middle-aged and old hearts. Endothelial and mesenchymal renewal is based on the two- and three-phase models, respectively.

See also Figure S3 and Tables S1 and S2.

constant cardiomyocyte number best follows the measured ^{14}C concentrations, and a >2.3-fold increase can be excluded (Figure S4).

Due to ploidization and cell renewal, most postnatal DNA synthesis in cardiomyocytes occurs during the first two decades of life (Figure 6A). Mathematical modeling revealed that postnatal cardiomyocyte turnover is highest in the first decade of life, declining to 0.8% per year at the age of 20 and to 0.3% per year at the age of 75, in the left ventricle (Figure 6B; Supplemental Experimental Procedures). With these dynamics, the difference between the youngest and oldest cells in a subject increases with age (Figure 6C). Most cardiomyocytes have already been generated perinataly, and approximately 80% of the cardiomyocytes will never be exchanged after 10 years of age, even during a long life. By 75 years of age, 39% of all cardiomyocytes have been generated postnatally, and of these, 36% are already generated by 10 years of age (Figure 6D). The turnover of right ventricle cardiomyocytes was not significantly different ($p > 0.05$, see the Supplemental Experimental Procedures) from the left ventricle with 50.4% of the cells being renewed by 75 years of age.

Our analyses reveal that the cell renewal in the heart is primarily confined to the endothelial and mesenchymal cell populations and that there is a much more limited exchange of cardiomyocytes.

DISCUSSION

The generation and cellular plasticity of the myocardium has been extensively studied during the last decade. However, differences in the estimates of the magnitude and time line of cardiomyocyte exchange have led to an ongoing debate about the capacity of the adult heart to renew cardiomyocytes. In this study, we combined several strategies, including ^{14}C birth dating and stereology, to provide a comprehensive view of myocardial renewal. We report that cellular remodeling of the myocardium is a lifelong process, with most postnatal cardiomyocyte exchange being restricted to the first decade of life.

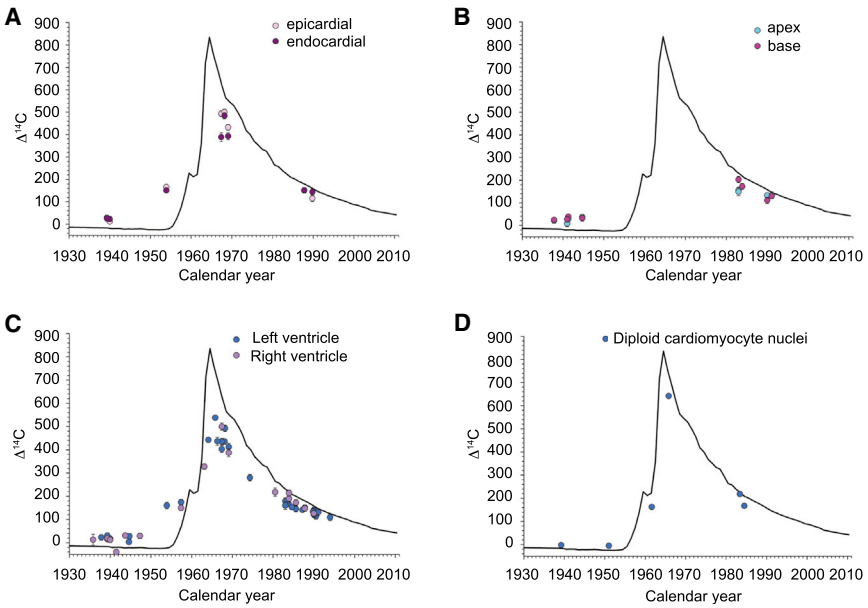

Figure 5. Retrospective ^{14}C Dating of Cardiomyocytes

(A–D) Genomic ^{14}C concentrations of cardiomyocytes in the human heart. (A to C) All ^{14}C concentrations from subjects born after the bomb spike correspond to time after the subjects' birth dates, indicating postnatal DNA synthesis. Most ^{14}C concentrations of subjects born before or during the time when the bomb curve peaks were elevated, indicating a DNA exchange even during adulthood. (A to C) Comparison between genomic ^{14}C concentrations of cardiomyocytes in the outer (epicardial) and inner (endocardial) myocardial layer (A), apex and base of the left ventricle (B), and the left (some ^{14}C data points were already reported by Bergmann et al. 2009; Figure S3E) and the right ventricle (C) revealed no significant differences between the populations (see Results). Most data points do not correspond to the ^{14}C concentration at birth, suggesting a limited postnatal and adult DNA turnover. (D) Genomic ^{14}C concentrations of diploid cardiomyocyte nuclei in the left ventricle indicate cardiomyocyte turnover. See also Figure S3 and Tables S1 and S2.

Endothelial and Mesenchymal Cell Compartments Are Highly Proliferative

Cardiac endothelial and mesenchymal cells expand during the physiological growth of the heart and show much higher generation rates than do cardiomyocytes. Endothelial cells have the highest exchange rate, with the whole population being renewed every 6 years in adulthood. It will be of clinical relevance how endothelial dysfunction and advanced arteriosclerosis as seen, for instance, in diabetic patients affect endothelial renewal. Mesenchymal cells show an age-dependent decline in renewal. One might argue that fibroblast proliferation is one of the hallmarks of interstitial fibrosis often seen in aging hearts, but hearts with signs of pathology were excluded from this study (see the Experimental Procedures).

Cardiomyocyte Generation in the Growing Human Heart

In rodents most cardiomyocytes exit the cell cycle within the first week postnatally as they become multinucleated (Walsh et al., 2010). In humans most cardiomyocytes remain mononucleated throughout life (Figure 1C). In agreement with earlier studies (Adler, 1991; Mayhew et al., 1997), we found no evidence that the physiological growth of the human heart is accompanied by the addition of cardiomyocytes. This finding is based on our stereological quantification (Figures 1A–1D), as well as by ^{14}C measurements (Figure S4), which exclude the possibility of a several fold increase in cardiomyocyte cell count as recently suggested by Mollova et al. (2013).

There is, however, substantial replacement of existing cardiomyocytes in growing hearts. Of all postnatally born cardiomyocytes generated up to 75 years of age, 36% are already generated by 10 years of age (Figures 6C and 6D). In the second decade of life, when the cardiomyocyte renewal rates approach adult levels, the DNA content per cardiomyocyte nucleus increases by ploidization, reaching adult levels at the age of 20 (Figure 1), indicating a switch from cytokinesis to polyploidization.

Most Cardiomyocytes Are Never Exchanged

The cellular plasticity of the adult human heart has been heavily debated. Analysis of genomic ^{14}C concentrations revealed that myocytes were on average 5 years younger than the subject in adulthood, and only 39% of the left ventricle cardiomyocytes are exchanged during a long life. ^{14}C dating of selectively diploid cardiomyocyte nuclei allows an assessment of renewal rates, without the need for taking polyploidization into account. This predicts a fraction of 47.8% that renews within a lifetime of 75 years. We note that the fraction is not directly comparable with that of the whole cardiomyocyte population because of the different cell-number kinetics; the diploid cardiomyocytes decrease in numbers during polyploidization. However, qualitatively, this is in good agreement with our model, including the whole cardiomyocyte population.

Most postnataly born cardiomyocytes are generated in young humans, which is in agreement with higher proliferation rates showed in young subjects (Mollova et al., 2013). Cardiomyocyte generation represents the exchange of cardiomyocytes without any increase in their number. The recently reported increase in cardiomyocyte number in juvenile mice could correspond to the reported ploidy increase in adolescent humans (Naqvi et al., 2014). Our data are in the range of most reported turnover rates in rodent models with renewal rates of 0% to 4.0% per year (Malliaras et al., 2013; Senyo et al., 2013; Soonpaa and Field, 1997; Walsh et al., 2010).

Cardiomyocytes Renew at Similar Rates in Different Subdivisions of the Human Heart

Studies in rodents have suggested that cardiac stem cells are not homogenously distributed (Cai et al., 2008). We asked

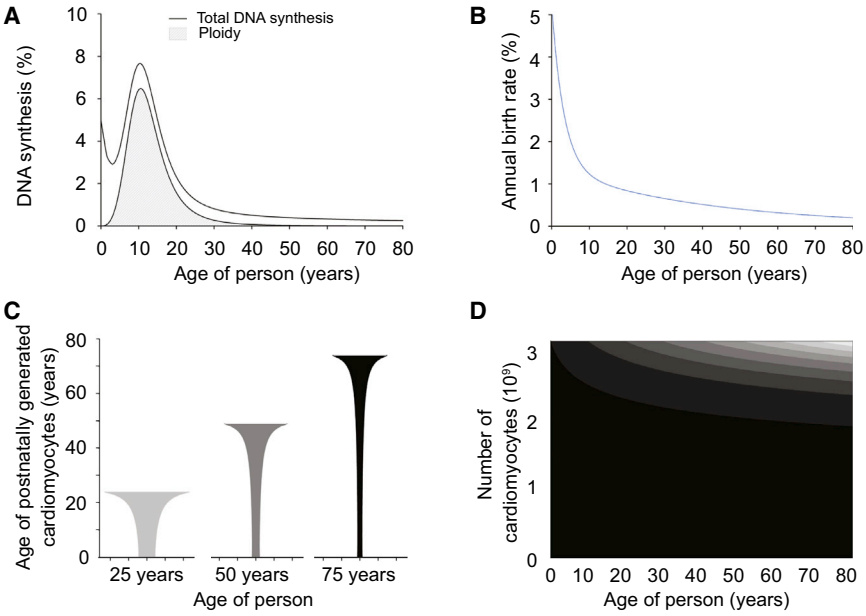

Figure 6. Renewal Dynamic of Cardiomyocytes in the Human Heart

(A) Total DNA synthesis inferred from genomic ^{14}C concentration in cardiomyocytes at different ages (black) with that associated with polyploidization indicated (pattern fill).

(B) Annual birth rates of cardiomyocytes in the left ventricle decline in an exponential fashion in the growing heart and adult heart. Cardiomyocytes renew at a rate of 0.8% at the age of 20, whereas in old subjects this rate declines to values below 0.3%.

(C) Age distribution of adult-born cardiomyocytes at 25, 50, and 75 years of age.

(D) The total number of cardiomyocytes is already established at birth and remains constant with age. Similarly as in Figure 4, for a given age of the person, postnatally generated cells are shown with different shades of gray, indicating the decade in which they were generated. During a lifetime, approximately 35% of all cardiomyocytes are exchanges postnatally, with most cardiomyocytes already generated at birth and in the first years of life. Cardiomyocyte renewal is based on the cumulative survival model.

See also Figure S4 and Table S3.

whether cardiomyocyte renewal might be heterogeneously distributed within the heart. Epicardial progenitor cells were shown to be re-activated after cardiac infarction by thymosin β4, an actin monomer-binding peptide that is involved in the development of the heart and generate new cardiomyocytes (Smart et al., 2011). However, we could not detect any differences in turnover rates comparing myocardial layers toward the epicardium compared to the myocardium facing the endocardium. Moreover, we could not find any differences in myocyte renewal comparing the apex with the heart basis, as one might have expected from studies reporting a higher frequency of stem cell niches in the apical regions in which the hemodynamic workload is lower (Beltrami et al., 2003). Finally, we asked whether turnover rates differ comparing the left ventricle with the right ventricle, both derived from different stem cell populations developmentally and exposed to substantially different workloads. Although the average DNA content per nucleus was significantly higher in the left ventricle, we could not find any differences in renewal rates between the left and the right ventricles. The homogeneity in myocyte renewal suggests that cardiomyocyte renewal is rather independent from ventricular configuration and hemodynamic workload.

In summary we have shown in this study that endothelial cells, mesenchymal cells, and cardiomyocytes are exchanged in the human heart during the entire lifespan, albeit with different magnitudes and dynamics. These findings suggest that it may be rational and realistic to develop new therapeutic strategies in order to augment endogenous regeneration to treat cardiac diseases.

EXPERIMENTAL PROCEDURES

Sample Preparation for ^{14}C Measurement

The right and left ventricle were dissected, removing epicardial fat and visible blood vessels. The left ventricle myocardium was further divided to obtain regions adjacent to the epi- or endocardium and from the apex and base. Isolation of cardiac cell nuclei was performed by mechanical breakdown of the tissue, followed by a series of filtering steps, and ultracentrifugation through a sucrose gradient. Isolated nuclei were stained with antibodies against pericentriolar material 1 and Ulex Europaeus Agglutinin I and sorted using fluorescence-activated cell sorting. DNA from sorted nuclei was extracted, and ^{14}C was measured as described in the Supplemental Experimental Procedures.

Stereological Analysis

Using a design-unbiased strategy, tissue biopsies (4 mm diameter) from a minimum of ten different regions of the left ventricle were sampled. The biopsies were embedded in 8% gelatin, and isectors (spheres) with a diameter of 5–6 mm were prepared to get isotropic, uniform random alignment of the samples. The isectors were embedded in cryostat embedding medium, and 40-μm-thick sections were stained for analysis for stereological quantitation (see subsection Immunohistochemistry). Analysis was performed on a LSM700 confocal microscope (63× and 40× Plan-Apo oil objective), using the ZEN2010b software with the newCAST module (v. 4) (Visiopharm A/S). A minimum of four isectors was sampled, and a minimum of 200 cells per subject was counted (1%–2% of the area of the region of interest). A systematic random sampling scheme (meander sampling) was applied using an optical disector with a counting frame (76 × 76 × 22 μm and 40 × 40 × 20 μm with 3 μm guard zones). To determine myocyte volume and the number of nuclei per myocyte, we defined local vertical windows at which myocytes have been cut along their longitudinal axis. Myocyte cell borders were labeled with connexin-43 (rabbit anti-connexin-43, 1:5,000, SigmaAldrich; pan-cadherin [mouse anti-pan cadherin, 1:200, Abcam] in infants), dystrophin (rabbit anti-dystrophin, 1:2,000, Atlas Antibodies), and myocyte nuclei with PCM-1. Cardiomyocyte volume was determined by the three-dimensional nucleator with five test lines. If more than one nucleus was present in a sampled myocyte, the first myocyte nucleus was used as reference point for the nucleator tool. Endothelial cells were identified with the lectin Ulex europaeus agglutinin I (UEA I) (Vector Labs), conjugated to fluorescein isothiocyanate. Mesenchymal cells were identified by the absence of PCM-1 and UEA I. To estimate the total numbers of cells in the heart, we utilized the two-step $N_V \times V_{REF}$ method (N_V is an estimate of the numerical cell density, and V_{REF} is the reference volume of the tissue or organ region of interest) using an optical disector (Brüel and Nyengaard, 2005). Reference volume (V_{REF}) was calculated from the

wet ventricular mass by applying the density of the myocardium 1.06 g/cm^3 (Brüel and Nyengaard, 2005). In case the whole ventricle was not available, we estimated the reference volume (V_{REF}) based on body surface area (BSA) (Figure S1B). When necessary, tissue shrinkage along the z axis was corrected. Tissue shrinkage along the x and y axes was not observed. The coefficient of error (CE) for most samples was less than 0.2 (20%).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.05.026.

AUTHOR CONTRIBUTIONS

O.B., S.Z., and J.F. conceived and designed the study. O.B., S.Z., A.F., K.A., and S.L.S. performed and analyzed the experiments. M. Salehpour and G.P. performed and analyzed accelerator mass spectrometry (AMS) measurements. S.Z. and S.B. performed mathematical modeling and statistical analysis. K.A., M.A., R.J., C. R., T.M, S.J., and H.D. procured the tissue and provided patient data. M. Szewczykowska and T.J. provided and interpreted pediatric echocardiography data. O.B. and J.R.N designed the stereological experiments. O.B., S.Z., and J.F. wrote the manuscript.

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