Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse

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Determination of cell types and numbers during cardiac development in neonatal and adult stages of rodents is of interest in development, homeostatic, and pathological stimuli. The aim of this study was to observe the alterations in myocardial cell populations during neonatal development in the adult animal and to observe any variations of the cellular components of the heart in different species, specifically, the rat and mouse. Whole hearts were isolated from either mice or rats during the neonatal and adult stages of development, and single cell suspensions were prepared via sequential collagenase digestion. Heterogeneous cell populations were immunolabeled for specific cell types and analyzed using fluorescence-activated cell sorting (FACS). In addition, the left ventricle, right ventricle, and septa were isolated, fixed, and sectioned for morphometric analyses. These studies enabled us to establish a homeostatic model for the myocardium that can be compared with genetic and cardiac disease models.

Cardiovascular cell populations were characterized using cell-specific molecular markers. The myocardium exhibits distinct regional differences influenced by the specific physiological nature of the region. In addition, the mouse model has become an ideal model for studying the heart through the use of various transgenic, knockout, and surgical models. However, there are specific physiological differences between the rat and mouse, such as heart rate and total collagen.

Moreover, the cell populations extrapolated from the early rat experiments have yet to be qualified in the mouse. Given this lack of knowledge, the relative heterogeneity of the myocardium, and the qualitative data demonstrating cardiac remodeling during early postnatal development, we decided to investigate fluctuations of cell populations in the adult and developing murine and rat hearts.

During normal function, cellular components of the heart interact in a dynamic fashion to respond to alterations in developmental, homeostatic, and pathological stimuli. The main cellular components that make up the heart include cardiac fibroblasts, myocytes, endothelial cells, and vascular smooth muscle cells (VSMCs), with the majority being fibroblasts and myocytes. These cell types maintain the electrical, chemical, and biomechanical responsive nature of the organ as well as the three-dimensional structure via autocrine, paracrine, and cell-cell interactions. Abrogation of these interactions via remodeling can cause interference in this system leading to pathological conditions. Thus, defining how these populations fluctuate during development and homeostasis can provide us with a better understanding as to how these cell populations are affected by pathological states.

Fluorescence-activated cell sorting (FACS) has been employed to measure individual cellular populations of a heterogeneous mix of cells based on their expression of specific molecular markers. In numerous studies, FACS analyses have become indispensable for identifying populations of lymphocytes and bone marrow-derived stem cells, and determining cellular percentages based on cell-specific marker expression that has been well established for these cell types. Recent studies have established specific markers for permanent cells of the myocardium in particular, cardiac fibroblasts, via discoidin domain receptor 2 (DDR2) and connexins (Cx40, Cx43, and Cx45) to function in electrical conduction in the heart. Abrogation of these interactions via remodeling can cause interference in this system leading to pathological conditions. Thus, defining how these populations fluctuate during development and homeostasis can provide us with a better understanding as to how these cell populations are affected by pathological states.

Studies in the 1970s from Zak (36) and Nag (26) established quantified relationships of cellular populations of the adult rat left ventricle based on morphological characteristics using electron microscopy of rat left ventricular sections as well as gradient centrifugation. These original studies on specific cardiac regions were extended to the whole heart and suggested that the heart is composed of 56% myocytes, 27% fibroblasts, 7% endothelial cells, and 10% vascular smooth muscle cells. Moreover, our morphometric and FACS data demonstrated similar percentages in the three regions examined. During neonate rat cardiac development, we observed a marked increase in numbers of cardiac fibroblasts and a resultant decrease in percentages of myocytes in late neonatal development (day 15). Finally, FACS analyses of the heart heart during development displayed similar results in relation to increases in cardiac fibroblasts during development; however, cell populations in the rat differed markedly from those observed in the mouse. Taken together, these data enabled us to establish a homeostatic model for the myocardium that can be compared with genetic and cardiac disease models.

Materials and Methods

Isolation of cardiac cells. Animals were humanely killed through cervical dislocation. This investigation conformed to the National Institutes of Health Guide for the Care Use of Laboratory Animals.

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Supernatant was removed, and cells were resuspended in Gey's Ca2+ buffer. Then centrifugated at 1,000 rpm for 5 min. The supernatant was then collected and pooled with that of prior steps. The pooled supernatant (containing isolated cells) was collected, and the remaining tissue was lyse any red blood cells. Cells were collected by centrifugation, resuspended in 1 ml FACS staining buffer (PBS without Mg2+, Ca2+), and count using a hemocytometer. Cells were then resuspended in a final concentration of 1 x 10^6 cells/50 uL for FACS analyses.

**Antibodies for FACS analysis.** Primary antibodies for the major cardiac cell types (cardiac fibroblasts, myocytes, endothelial cells, and VSMCs) were chosen based on current literature (5, 7, 9, 15, 21, 23, 27). Respectively, antibodies against DDR2 (N-20, Santa Cruz Biotechnology), α-MHC (ab15, Abcam), α-SMA (MAB 1420, R&D Systems), and CD31 (37-0700, Zymed) were conjugated to quantum dots as described by the manufacturer (Invitrogen). Briefly, inactive amine groups on the surface of the quantum dots were modified to thiol-reactive maleimide groups. Reactive quantum dots were then conjugated to antibodies that had undergone reduction of several disulfide bonds. Excess dots or unconjugated antibodies were then removed via size-exclusion chromatography. We observed our antibody conjugation efficiency to be ~60–70% with final concentrations ranging from 0.3 to 0.4 μg/μL of antibody conjugate.

**Cell staining and general cell sorting.** Cell populations were analyzed using the BD Cytotoxic/Cytoperm kit (BD Bioscience Pharmingen, San Diego, CA) as described by the manufacturer. Briefly, cells were harvested as described above and stained for DDR2, CD31, α-MHC, and α-SMA. Antibodies against cell-surface markers (DDR2 and CD31) or against internal cell markers (α-MHC and α-SMA) were incubated for 30 min. For internal cell markers, cells were permeabilized using BD-cytofix/cytoperm solution for 20 min at 4°C and then incubated with antibodies. Cells were washed, resuspended in FACS staining buffer, processed using the Epics XL FACS, and analyzed using Expo 32 software (Beckman Coulter, Miami, FL).

**Morphometric analyses.** Hearts were frozen in fresh 4% paraformaldehyde and frozen in OCT, and 25-μm serial sections were cut. Tissue autofluorescence was quenched by incubation of the sections sequentially in PBS-glycine (150 mM) followed by NaBH4 (1 mg/ml in PBS). Myocytes were labeled with a mouse monoclonal antibody to α-MHC (Chemicon, Temecula, CA) and visualized with Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA); fibroblasts were labeled with anti-DDR2 (Santa Cruz Biotechnology) antibody or anti-vimentin (Chemicon) antibody and visualized with Cy2 conjugated to donkey anti-chicken IgG (Jackson ImmunoResearch); and nuclei of all cells were labeled with 4',6-diamidino-2-phenylindole (DAPI). From the left ventricle, right ventricle, and intraventricular septum of each section, 20–40 consecutive, nonoverlapping confocal images of the 3 fluorescence probes were acquired in a single optical plane using an AtoFluor CARV confocal system on a Zeiss Axiovert 200M inverted fluorescence microscope, and images were captured using AQM Advance-6 acquisition software (Kinetic Imaging, South Windsor, CT). Numbers of myocytes, fibroblasts, and other cell types in each section were determined using MetaMorph Image Analysis software (Molecular Devices, Sunnyvale, CA), and statistical analysis was carried using SigmaStat (SPSS, Chicago, IL) software with P < 0.05 considered significant.

From the DAPI image, numbers of nuclei in the section were determined using the integrated morphometry subroutine of MetaMorph. The high-contrast image of DAPI-stained nuclei was overlaid with the α-MHC-positive Cy3-stained image (myocytes). Colocalization of Cy3 and DAPI gave the total numbers of myocytes in the image. Similarly, the image of DAPI-positive nuclei was overlaid with the DDR2-positive Cy2-stained image or vimentin-positive Cy2-stained image (fibroblasts), and colocalization of Cy2 and DAPI gave the total numbers of fibroblasts in the image. Nuclei that did not colocalize with either Cy3 or Cy2 were defined as other cell types (endothelial cells, VSMCs, etc.). Cell counts from 20–40 consecutive nonoverlapping images were averaged to give representative numbers of the populations of these cells in the morphological region of interest.

**FACS analyses** were performed on isolated age-matched hearts as described above. Hearts were isolated and divided into the left ventricular free wall, right ventricular free wall, and intraventricular septa. These sections were then digested, labeled, and subjected to FACS as described above.

**Neonatal rat bromodeoxyuridine staining.** Cell proliferation was measured using bromodeoxyuridine (BrdU) incorporation using the cell-proliferation assay kit BrdU/IFA (Roche, Indianapolis, IN) as described by the manufacturer. Briefly, neonatal rats at 3, 5, 10, and 13 postpartum were injected with BrdU and then killed 5 h later, and hearts were extracted. Hearts were snap frozen in OCT, and 25-μm serial sections were cut. Samples were stained with DAPI and imaged using a Zeiss LSM 510 Meta confocal microscope. Samples were imaged for DAPI and BrdU-FITC, and samples that labeled positive for both DAPI and BrdU-FITC were considered to be proliferating. Cell counts from 10–20 sections were averaged to give representative numbers of proliferating cells.

**Hydroxyproline assay.** To determine the total collagen content in rat and mouse hearts, we performed a modified colorimetric hydroxyproline assay as previously described (12). Briefly, whole hearts were isolated from adult animals, and the ventricles were separated from the atria. Samples were then freeze dried overnight in a LabConco Freeze Dryer 3 (LabConco, Kansas City, MO). Samples were then weighed and hydrolyzed overnight with 6 N HCl at 120°C. For a standard curve, 2–70 μg of hydroxyproline were added to individual wells in a 96-well plate. Unknown hydrolyzed samples were then added to separate wells, and samples incubated with Ehrlich reagent (p-dimethylamino-benzaldehyde) for 30 min at 80°C. Samples were then read using a Bio-Rad Benchmark Plus plate reader at 557 nm. Calculations of hydroxyproline levels were then calculated against the known standards. Total hydroxyproline per milligram of total heart was then calculated.

**RESULTS**

**Adult murine cell populations.** To define the cell populations of the heart, we first examined the adult murine heart using cell-specific markers for each of the major constituents of the heart and performed detailed FACS analyses. Figure 1 shows a representative FACS experiment with unstained cells serving as a negative control (Fig. 1A, top left) and composite data from six animals (Fig. 1B). Approximately 27% of the cardiac cells were DDR2 positive (fibroblasts), whereas 56% of the cells were α-MHC positive (myocytes) (Fig. 1B). In addition, we observed that 7% of cells were CD31 positive (endothelial cells) and 10% were α-SMA positive (VSMCs) (Fig. 1B). These observations of the cellular populations of the heart were

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markedly different from the 70% nonmuscle and 30% muscle cell numbers obtained from studies (26, 36) involving the rat left ventricle. Therefore, these data suggest that variations in cell ratios may exist between species as well as in various regions of the heart.

Mouse and rat cell populations during development. Several studies (18, 31) have reported that in normal hearts, the transition from fetal circulation to postnatal circulation is characterized by increases in blood pressure and left ventricular size. Interestingly, elevated blood pressure and left ventricular hypertrophy are also associated with cardiac pathologies. To examine this issue of proliferative capacity of the heart, we performed BrdU staining on sections from the neonatal rat heart (Figs. 2, A–I, and 3A). On neonatal day 3, we observed that the number of BrdU-positive cells was ~37%, whereas by neonatal day 5, the number of BrdU-positive cells decreased to ~11%. This downward trend continued throughout development, as indicated by only 5% BrdU-positive cells in a neonatal day 13 heart (Figs. 2, A–I, and 3A). These data suggest that the early neonatal period is initially characterized by elevated cellular proliferation, which progressively decreases as the heart becomes normalized to adult blood pressure and volume.

We next sought to observe any corresponding cell population changes in the developing rat heart using our FACS technique. In the day 1 rat heart, we observed that the cardiac fibroblast population was ~30% of the whole heart and the myocyte population comprised ~62% of the heart with 8% of the heart consisting of the remaining nonmyocyte, nonfibroblast populations (Fig. 3B). We then examined the rat cardiac cell populations at day 15 and observed that cardiac fibroblasts made up ~64% of the total heart, whereas the myocyte population was reduced to 30%, with the nonmyocyte and nonfibroblast cell populations comprising the remainder of the heart (Fig. 3B). These cell population numbers are similar to those observed in the original studies of the rat left ventricle by Zak and Nag (26, 36). Taken together, these data suggest that there is a dramatic difference in cell populations of the heart between various species (26, 36).

Having observed the alterations of cardiac populations in the developing rat heart, we next examined the developing murine heart. We performed FACS analyses to examine the fluctuations in the relative cell populations of the murine heart on embryonic day 18.5 and neonatal days 1, 5, and 15. At embryonic day 18.5, the cardiac fibroblast population was observed to be 14% of all cardiac cells (Fig. 4). By day 1, cardiac fibroblasts had decreased to only 10% of the cardiac cell population, whereas by day 5, the percentage of fibroblasts increased to 14%, a relative increase of 27% during the first 5 days of postnatal life (Fig. 4). By day 15, the percentage of fibroblasts was ~18%, a relative increase of 24% over the previous 10 days. Additionally, cardiac myocyte percentages fluctuated during the observed neonatal period, particularly between days 1 and 5 and between days 5 and 10. Moreover, the observed adult percentage of cardiac myocytes was significantly lower than neonatal percentages (Fig. 4). Furthermore, the percentages of nonmyocyte/nonfibroblast cells fluctuated from 20% to 29% in early embryonic and neonatal periods. While these cell numbers were reduced in the latter neonatal period relative to the increase in fibroblasts, the percentages did increase relative to the reduction of myocytes.

Regional adult cell populations/morphometric analysis. To better evaluate individual cell populations of the adult murine heart, we performed morphometric analyses on the left ventricular free wall, intraventricular septum, and right ventricular

Fig. 1. Adult total heart murine FACS analyses. A: representative FACS analyses of a 12-wk-old adult mouse heart. The top left is a negative control sample. Note that the mean fluorescent intensity is below the first decade. In subsequent samples, all events with mean fluorescent intensity above the 10th decade are considered as positive for the immunofluorescent label. α-MHC, α-myosin heavy chain; DDR2, discoidin domain receptor 2; α-SMA, α-smooth muscle actin. B: composite average of cardiac cell populations from all experiments. Cardiac fibroblasts were ~27%, myocytes were ~56%, endothelial cells were ~7%, and vascular smooth muscle cells (VSMCs) were ~10%. N = 6.
free wall (Fig. 5A). These data were then used to corroborate FACS analyses of the same regions (Fig. 5B). Figure 5A shows representative staining of the left ventricular wall with DDR2 (green), α-MHC (red), and DAPI (blue). As shown in Fig. 5B, the left ventricular free wall consisted of ∼52% myocytes and 37% fibroblasts, with the remaining 11% consisting of other cell types as determined by morphometric analyses. These data did not display statistical significance from our data obtained via FACS, demonstrating that the left ventricular free wall consists of ∼51% α-MHC-positive cells (myocytes), 37% DDR2-positive cells (fibroblasts), and a 12% nonmyocyte/nonfibroblast population (Fig. 5B). Data from the intraventricular septum and right ventricular free wall also demonstrated no significant differences between data obtained either via morphometric or FACS analyses (Fig. 5B).

**Hydroxyproline content of rat and mouse hearts.** To assess the fluctuations in collagen deposition in the rat and mouse heart, we performed hydroxyproline analyses (Table 1). These experiments revealed that adult rat hearts contain significantly more hydroxyproline ($P < 0.01$) than hearts isolated from adult mice (Table 1). These data, as well as the literature, indicate that the larger the mammal, the greater the collagen content of the heart. This increase in collagen content corresponds to the observed increase in cardiac fibroblasts present in rat hearts compared with murine hearts (Table 1; $P < 0.01$).

**DISCUSSION**

**Mouse versus rat adult cell populations.** The overall goal of this study was to define cell populations in the adult and
the cellular constituents of the heart plateau and thus give us a heart that was more characteristic of the adult. We observed that the early neonatal period was characterized by ~33% BrdU-positive cells, with significant decreases from days 3 to 5 and from days 5 to 10 (Figs. 2, A–I, and 3A). Days 10–13 did not show any significant changes in numbers of BrdU-positive cells. Thus, we approximated day 15 to be when we would observe cardiac cell populations in the rat similar to those observed in the adult. Our FACS data demonstrated that the number of myocytes was ~30%, the number of fibroblasts was 64%, and nonmyocyte/nonfibroblast cell populations made up the remaining 6% of the rat heart. These cell ratios were similar to those observed by Zak and Nag in their original studies on cell populations of the rat left ventricle. Moreover, we examined early (day 1) neonatal cell populations and observed that the rat myocardium at day 1 was comprised primarily of cardiac myocytes (~62%), with cardiac fibroblasts making up the largest portion of the nonmyocyte population (~30%). These ratios were significantly ($P < 0.001$) altered from the day 15 rat heart. Taken together, these data suggest that there is a striking species difference in the makeup of cellular constituents of the heart in the rat and mouse (Table 1).

These findings are not entirely surprising considering that there are significant differences between the two species’ (rat and mouse) physiological cardiac parameters. For example, the mouse heart has a heart rate of 500–700 beats/min, whereas the rat has a heart rate of 300–400 beats/min. These differences in physiological parameters suggest that the myocardial cell composition is responsive to the input and that this is the causative factor for the observed species differences. One possible explanation for this can be found in the law of Laplace. The law of Laplace states that the larger a vessel’s radius, the larger the wall tension required to withstand a given developing rat and murine heart through the use of cell-specific markers, FACS analyses, and confocal microscopy. Transgenic and knockout mice have become the standard model for a wide array of human diseases. Given the lack of data specific to the murine myocardium’s quantified cell populations, our first goal was to define adult cell percentages of the mouse heart and to compare our FACS data against known data from the rat heart. Using FACS analyses, we observed the adult murine heart to consist of ~56% cardiac myocytes, 27% fibroblasts, 7% endothelial cells, and 10% VSMCs (Fig. 1B). These data differ significantly from the original studies of Zak and Nag in their original studies on the rat heart. Using FACS analyses, we observed the rat left ventricle as being composed of 30% muscle and 70% nonmuscle cell populations (7, 9, 26, 36). Since these data deviated from the accepted literature and the regional nature of the heart, we analyzed the rat heart to examine cellular populations and to compare these with the cellular percentages observed in the mouse heart.

Given the general difficulty in isolating single cells from a solid mass as large as the adult rat heart, we first performed BrdU analyses on isolated sections of the neonatal rat heart. The neonatal heart has been characterized qualitatively by an increase in interstitial cell numbers via hyperplasia and myocyte cell size via hypertrophy (7, 9, 35). As the heart becomes more like the adult heart, the two processes of expansion are decreased. As such, BrdU staining would allow us to determine the exact day in postnatal development when the hyperplasia of...
internal pressure (6). Alteration in the radius of the heart in a transventricular tension model designed to reduce the radius of curvature of the left ventricle has been shown to alter hemodynamic function and wall thickness (25). Given that the radius of curvature of the left ventricle of a rat is larger than that of a mouse, the law of Laplace suggests that the wall tension on the heart would increase in the rat versus the mouse (6). This indicates that the rat would need an increased amount of connective tissue and thus an increase in the number of fibroblasts and/or a different cellular composition based on the radius of the heart given equal pressures. The suggestion that an increase in wall tension is the causative factor for the difference is indicated by several studies (11, 13, 22) that have demonstrated that the mean arterial pressure in the mouse (101100108 mmHg) is almost identical to the rat (101100106 mmHg). Thus, an increase in tension would require an overall increase in connective tissue and interstitial cells. Concurrent with this hypothesis, our data indicate that there are increased numbers of fibroblasts in the rat compared with the mouse. Therefore, the assumed conclusion is that the amount of extracellular matrix in the heart would be greater in the rat than in the mouse, indicating an increase in tension on the heart. This can be seen in studies assaying for hydroxyproline content in the heart (12). Collagen is unique in that it is one of the few proteins that contains hydroxyproline (8, 12, 34). To examine total collagen levels in the heart, we performed hydroxyproline analyses of both adult rat and mouse hearts. Our analyses revealed that the adult rat heart contained more hydroxyproline compared with the mouse, 9.36 versus 5.95 µg collagen/mg dry heart wt, respectively (Table 1). As observed in our study, this also corresponds to an increase in cardiac fibroblast numbers in the rat heart (Figs. 1B and 3B and Table 1). To our knowledge, this is the first study to correlate differences in the number of cardiac fibroblasts between species and an increase in the amount of collagen.

Taken together, these data indicate that larger mammals, which all display similar mean arterial pressures, would have greater wall tensions given their increased radius of curvature. Indeed, a survey of previous studies examining the differences between larger and smaller mammals demonstrated an increase in hydroxyproline content as seen in a comparison between rats and dogs (20). As in our study, this comparison of large and small mammals reveals that as the radius of curvature of the left ventricle increases, so does the extracellular matrix, which offsets the increase in wall tension. Thus, given that cardiac fibroblasts are the primary producers of collagen in the heart, the rat heart may in fact require more fibroblasts to provide the means to compensate for the increase in wall tension (7, 9). Therefore, these data, as well as those of previous studies, suggest that there are species differences between the rat and mouse in regard to cellular constituents and that the data of Zak and Nag may only apply to the rat left ventricle and not to other species.

Table 1. Hydroxyproline content and comparative adult cell populations in adult rat and mouse hearts

<table>
<thead>
<tr>
<th>Species</th>
<th>Hydroxyproline Content, µg collagen/mg dry heart wt</th>
<th>Cardiac Fibroblasts, %</th>
<th>Cardiac Myocytes, %</th>
<th>Nonmyocytes/Nonfibroblasts, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>9.36 ± 0.71</td>
<td>62.6 ± 1.5</td>
<td>26.4 ± 5.8</td>
<td>4.9 ± 3.1</td>
</tr>
<tr>
<td>Mouse</td>
<td>5.95 ± 0.28*</td>
<td>26.1 ± 5.7*</td>
<td>55.9 ± 8.3*</td>
<td>17.6 ± 4.0*</td>
</tr>
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Values are means ± SE. *P < 0.05 vs. values for the rat.

Fig. 5. Morphometric and FACS analyses of distinct cardiac regions. A: 25-µm sections were stained with DAPI for nuclei (top left), vimentin for fibroblasts (Fibro; top right), and α-MHC for myocytes (Myo; bottom left). The bottom right demonstrates an overlay of the three stains from the same section. DDR2 was also used in these analyses, and no significant differences were observed in fibroblast numbers between DDR2-stained sections and vimentin-stained sections (data not shown). B: graphical representation of the left ventricular free wall, intraventricular (IV) septa, and right ventricular free wall by morphometric and FACS analyses on respective sections. No significant differences were observed between FACS and morphometric analyses.
Cardiovascular cell populations during neonatal development. The early neonatal myocardium undergoes several key changes during the switch from fetal to neonatal circulation that affects the structure of the myocardium, changes such as decreased pulmonary vascular resistance, increased pulmonary venous return, increased systemic resistance, and closure of the ductus arteriosus and foramen ovale (2, 37). Qualitative and quantitative developmental studies (31, 37) have demonstrated that during this transition from fetal to postnatal circulation, the heart undergoes an increase in pressure and volume, primarily from closure of the ductus arteriosus and a subsequent increase in flow to the left ventricle from the pulmonary circulation. This increase in pressure and volume contributes to an increase in the overall size of the organ (18, 31, 37). As observed in these studies, the left ventricle of the early neonate is under equal to lower pressure and flow compared with that of the right ventricle. At the cellular level, several studies (2, 35, 37) have qualitatively defined the cell populations in this neonatal phase as dynamically fluctuating during early neonatal growth. The majority of these studies defined nonmyocytes as increasing due to hyperplasia and myocytes increasing via hypertrophy, although some of these studies observed some myocyte proliferation in the heart (3, 7, 9, 10, 32, 35). These data suggest that the cellular ratios change in a spatial and temporal manner with respect to the dynamic biomechanical, electrical, and chemical input to the heart. Our data provide a quantification of cellular population changes in the heart. Moreover, this study demonstrates that the cellular populations of the heart change throughout the lifespan of the rat and mouse.

Given these previous data, we explored remodeling of the heart during the early neonatal and late embryonic periods. During the early neonatal period, we observed marked decreases in percentages of BrdU-positive cells as the animal aged (Figs. 2, A–I, and 3A). These data suggest that the heart responds to the increase in biomechanical load and signals in a dynamic manner that alters the cellular populations of the heart. In addition, our data exploring the developing rat myocardium demonstrated significant cell population changes from days 1 to 15. Having observed the dramatic changes in the various cell populations, we chose to further analyze this period to observe cellular population fluctuations in the developing mouse myocardium.

Our FACS analyses of the late embryonic heart demonstrate that cardiac fibroblasts levels are higher than those observed in the early neonate (Fig. 4). In the day 1 neonatal mouse, we also observed an increase in nonmyocyte/nonfibroblast cell populations. This suggests that the physiological changes from the fetal to newborn circulation causes developmental changes in the coronary vasculature. However, this hypothesis requires further examination in future studies. We also observed a slight decrease in myocytes; however, this reduction in myocyte percentage was not statistically significant.

In the rat heart, we observed that during the course of neonatal rat development, numbers of BrdU-positive cells were high in the first few days of neonatal life but markedly lower at latter time points (Fig. 3A). In addition, previous studies (18, 31) have demonstrated a large increase in neonatal development but a slower response later in development. Several qualitative studies (3, 7, 9, 10, 32, 35) have also indicated that the cells of the interstitium increase via hyperplasia. Indeed, cardiac fibroblasts have been characterized as cells that respond to an increase in biomechanical load in a proliferative and functional manner (7, 9, 15). Here, we demonstrated that the increase in pressure previously observed in the neonatal period affects fibroblast numbers by increasing the relative cellular percentages from days 1 to 15. Moreover, we observed that the changes in cardiac fibroblast numbers also have an effect on the other cell populations of the heart (Fig. 4). Myocytes in the mouse displayed an increase in relative percentages from days 1 to 5 but a decrease during the progression from the neonatal period into adulthood (Fig. 4). The increase seen in myocytes during the first days of life in the mouse correlates with observations made by Soonpaa and colleagues (29). In the mouse, Soonpaa et al. (29) observed an increase in DNA synthesis in myocytes during the first days of life, peaking at day 4.6. This increase in DNA synthesis in the early neonate did not correspond with an increase in the percentage of binucleated cells, suggesting that this can be attributed to an increase in the number of total cells. In the latter phase of neonatal life, days 7–15, the increase in DNA synthesis was coupled with a marked increase in binucleated cells. These data are again concurrent with our work, which does not show an increase in the total numbers of myocytes, and instead we observed a decrease in the percentage of myocytes in the heart relative to the increase in cardiac fibroblasts. Furthermore, the nonmyocyte/nonfibroblast cell population also decreased from days 1 to 5 and stayed constant until a modest increase was observed in adulthood. Thus, we observed dynamic cell population fluctuations in a novel manner in response to the increase in biomechanical load and developmental factors in late embryonic and neonatal periods and into adulthood.

Morphometric and FACS analyses of the left ventricle, right ventricle, and intraventricular septum. To isolate single cell populations from a solid tissue, the tissue itself must be disrupted. Given the difficulty in isolating the heart into a single cell suspension, we performed a double-blind study on the left and right ventricular free walls and intraventricular septum using both FACS analyses and traditional confocal microscopy and cell population analysis software to examine cell populations of the heart.

Previous studies (7, 10, 14, 16, 30) have demonstrated that different regions of the heart exhibit differences in the ratio of fibroblasts to myocytes, such as the sinoatrial node as opposed to the left ventricular free wall. This complex regional heterogeneity at the cellular level accounts for the complex nature of the heart as a whole. Indeed, several studies (7, 9, 10, 19, 21) have demonstrated that alterations of the local cellular makeup can alter regional cardiac function and thus abrogate the proper function of the entire heart. Given the relative heterogeneity of the myocardium, we expected to see significant differences in the regions chosen in the heart. However, we did not observe significant heterogeneity during our analyses of the left ventricular free wall, intraventricular septa, and right ventricular free wall when the three regions were compared (Fig. 5B). This is not surprising given that studies (7, 9, 14, 31) in the rabbit myocardium have demonstrated a common distribution of Cx43 and Cx45 and that the functional nature of the regions is similar to one another.

In addition, we also observed that analyses done via traditional staining and confocal microscopy did not differ significantly from the data obtained via FACS (Fig. 5B). Therefore, we have provided validation for our cell sorting technique and
findings in the cellular composition of the developing and adult murine and rat hearts. We used both DDR2 and vimentin in our morphometric and FACS analyses, as both of these markers have been shown to be useful in identifying fibroblasts in tissue (7, 9, 10, 15, 24). We did not observe any difference in our confocal analyses of fibroblasts between DDR2-stained sections and vimentin-stained sections (Fig. 5A and data not shown). One caveat is that our morphometric data do not take into account the concept of binucleation, which has been observed in cardiac myocytes in both the rat and mouse (1). Soonpaa and colleagues (29) observed that during the perinatal period, mouse myocytes undergo increased karyokinesis, ultimately resulting in ~85% of adult myocytes being binucleated. Gerdes et al. (20) observed a similar pattern of binucleation in the rat myocardium. However, these studies differed in the exact numbers and kinetics of myocyte binucleation when compared with a study from Anversa et al. (4), which showed that the percentage of binucleation in the rat is ~46%. Species differences have also been observed with the human heart ranging from 25% to 57% and levels of myocyte binucleation being at only 32% in the pig (1). While our results did not display a significant difference in cell population numbers obtained via FACS or morphometry, we did observe a modest standard deviation in the numbers of myocytes per field in our morphometric analysis. These data indicate that some of the myocytes counted may have been binucleated. Due to the difficulty in distinguishing mononucleation from binucleation in confocal analyses and the variability in the literature as to the exact percentage of binucleation, future FACS analyses would be prudent to analyze the exact percentage of binucleated myocytes.

In the present study, we examined cardiac cell population fluctuations during murine and rat development and presented a novel and rapid method of analyzing cell populations of the heart using FACS. The data presented in this study demonstrate the relative percentages of distinct, definable cell types present in the fetal, neonatal, and adult hearts as well as the effect that homeostasis may play in regulating cell specification. These data also demonstrate that there are significant species variations in the cellular makeup of the heart and suggest that caution should be taken when extrapolating cell population changes and comparisons between rat, mouse, and human hearts. Overall, the understanding of alterations in the regional and developmental cellular architecture throughout the life of the heart can give us a basis to address several critical questions regarding perturbations of developmental signals and/or pathological conditions that alter the normal function of the heart.

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