Endocardium Contributes to Cardiac Fat

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ABSTRACT

**Rationale:** Unraveling the developmental origin of cardiac fat could offer important implications for treatment of cardiovascular disease. The recent identification of the mesothelial source of epicardial fat tissues reveals a heterogeneous origin of adipocytes in the adult heart. However, the developmental origin of adipocytes inside the heart, namely intramyocardial adipocytes, remains largely unknown.

**Objective:** To trace the developmental origin of intramyocardial adipocytes.

**Methods and Results:** In this study, we identified that the majority of intramyocardial adipocytes were restricted to myocardial regions in close proximity to the endocardium. Using a genetic lineage tracing model of endocardial cells, we found that Nfatc1+ endocardial cells contributed to a substantial number of intramyocardial adipocytes. Despite the capability of the endocardium to generate coronary vascular endothelial cells surrounding the intramyocardial adipocytes, results from our lineage tracing analyses showed that intramyocardial adipocytes were not derived from coronary vessels. Nevertheless, the endocardium of the postnatal heart did not contribute to intramyocardial adipocytes during homeostasis or after myocardial infarction.

**Conclusions:** Our in vivo fate-mapping studies demonstrated that the developing endocardium, but not the vascular endothelial cells, gives rise to intramyocardial adipocytes in the adult heart.

**Keywords:** Lineage tracing, intramyocardial adipocytes, endocardium, vascular endothelial cells, endothelial cell, endothelial cell differentiation.

**Nonstandard Abbreviations and Acronyms:**

- EMT: Endothelial-to-mesenchymal transition
- VECs: Vascular endothelial cells
- MI: Myocardial infarction
- EPDCs: Epicardial derived cells
- Nfatc1: Nuclear factor of activated T cells
- PLIN: Perilipin 1
- TNNI3: Troponin I, cardiac 3
- FABP4: Fatty acid binding protein 4
- CDH5: Cadherin 5
- PECAM: Platelet/endothelial cell adhesion molecule 1
- RFP: Red fluorescence protein
- IRES: Internal ribosome entry site
- PPARg: Peroxisome proliferator activated receptor gamma
- CEBPa: CCAAT/enhancer binding protein (C/EBP), alpha
- UCP1: Uncoupling protein 1
- EMCN: Endomucin
- Apln: Apelin
- aMHC: Myosin heavy chain 6, cardiac muscle, alpha
- CTRP9: C1q and tumor necrosis factor related protein 9
INTRODUCTION

Over 64% of adults in the United States and more than half of the population in other developed countries are overweight or obese. Obesity is among the most significant risk factors in development of common medical conditions including type 2 diabetes, cardiovascular diseases, stroke and fatty liver disease. Given that obesity has become an epidemic and obesity-associated cardiovascular complications contribute to one of the principal causes of death, there has been a growing interest in understanding the developmental origins of adipose tissues. Indeed, recognition of cellular ontogeny would provide a necessary context to address issues associated with fat tissue metabolism, cardiovascular diseases, as well as cardiac repair and regeneration.

Fat not only stores up in subcutaneous tissues, but also accumulates in ectopic sites such as heart, liver and muscle, causing some organ-specific diseases. In the heart, the most obvious fat can be found in the epicardium, which forms epicardial adipose tissues. Recent studies based on genetic lineage tracing strategy revealed epicardium as the developmental origin of these epicardial fat cells. Furthermore, the adult epicardial cells could be reactivated and respond to cardiac injury, giving rise to new adipocytes during myocardial infarction. In addition to the epicardial location, fat could also accumulate deep inside the myocardium, which was regarded as intramyocardial adipocytes. Excessive intramyocardial fat depots are associated with many cardiovascular diseases such as cardiac lipotoxicity, arrhythmogenic right ventricular cardiomyopathy, left ventricular tachycardia after myocardial infarction, inflammation, atherosclerosis and heart failure. Nevertheless, it remains largely unknown about the developmental origin of the intramyocardial adipocytes. Therefore, understanding the cellular ontogeny of the intramyocardial fat tissue is not only important for developmental biology, but also contributes direct implications on novel treatment options for cardiovascular diseases.

Endocardium is a layer of specialized endothelial cells that cover the trabecular myocardium in developing heart. Endocardium represents a unique cardiac lineage derived from Flk⁺ multipotent cardiovascular progenitors. During cardiac valve development, endocardial cells undergo endothelial-to-mesenchymal transition (EMT) to form endocardial cushion, which is essential for the formation and remodeling of valves. In addition to valve mesenchymal cell contribution, endocardial cells could also give rise to coronary vascular endothelial cells (VEC). However, it is still controversial over the magnitude by which the endocardial cells contribute to coronary vessels in the developing heart. Recent study showed that endocardial cells contribute to coronary vessels in ventricular septum and the inner myocardial wall during late embryonic and early neonatal stages. These studies, albeit with difference in quantitative and temporal contribution, confirmed the involvement of endocardium in coronary angiogenesis. It has been reported that the vascular endothelium gives rise to both white and brown adipocytes, suggesting a model to study coordination of adipogenesis and angiogenesis during adipose tissue expansion. Indeed, development of adipose tissues is spatially and temporally associated with angiogenesis, indicating the possible involvement of coronary angiogenesis during intramyocardial adipocytes formation.

In this study, we reported that most intramyocardial adipocytes were restricted to myocardial regions in close proximity to the endocardium. Genetic lineage tracing analyses showed that the endocardial cells contribute to both intramyocardial adipocytes and coronary VECs. Our fate mapping studies also revealed that endocardial cells, but not VECs, give rise to intramyocardial adipocytes in the adult heart. The identification of endocardial source of intramyocardial fat will enable further studies on the biochemical and physiological cues regulating fat tissue formation inside the heart.
METHODS

Detailed materials and methods are provided in the Online Data Supplement. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. Immunostaining and in situ hybridization were performed according to protocol described previously. All data were presented as mean values ± SEM. Statistical comparisons between data sets were done by a two-sided unpaired Student’s t test for comparing differences between two groups. \( P < 0.05 \) was considered to be statistically significant.

RESULTS

Intramyocardial fat tissues are more restricted to the myocardium facing towards the endocardium.

To understand where the intramyocardial adipocytes located, we performed immunostaining for the lipid droplet-associated protein, perilipin (PLIN) on heart sections of mice at different stages. We collected hearts at embryonic day (E) 15.5, postnatal day (P) 0, 1 week (P1w), P2w, P3w, P4w and P8w. Immunostaining of PLIN showed that both intramyocardial and epicardial fat tissues appeared at approximately P3w to P4w, and the number of PLIN+ cells increased at P8w (Figure 1A and Online Figure IA). We confirmed this phenotype of intramyocardial adipocytes by oil red staining (Online Figure IB). In P26w adult heart, these intramyocardial oil red+ adipocytes were in close proximity to the innermost layer of the endocardium with some entirely attached to the endocardium (Figure 1B). In addition, co-staining of PLIN and the cardiomyocyte-specific marker TNNI3 revealed that PLIN+ adipocytes in the epicardial layer were restricted to atrioventricular groove (Figure 1C, arrowheads), whereas the intramyocardial PLIN+ adipocytes lied among cardiomyocytes located in close proximity to the endocardium (Figure 1C, and arrows in 1D).

We also noticed that some intramyocardial adipocytes were not located in close proximity to the innermost layer of endocardium facing the ventricular chamber, but they reside close to the endocardium that lines between two blocks of myocardium (Online Figure II). To address whether the endothelial cells lining between the two blocks of myocardium were indeed part of the endocardium, we used the coronary VEC-specific marker FABP4 to distinguish coronary VECs (FABP4+CDH5+) from endocardium (FABP4-CDH5-). The endothelial cells lining between the two blocks of myocardium were FABP4+CDH5+ endocardial cells, and intramyocardial adipocytes were located next to them (Online Figure IIA,B). Similarly, we used an additional coronary VEC-specific marker apelene (APLN) to distinguish endocardial cells (APLN-PECAM+) and coronary VECs (APLN+PECAM+). We found that a subset of intramyocardial adipocytes were located close to these endocardial cells that lined between the two blocks of myocardium (Online Figure IIC,D). Quantification of the distance between the PLIN+ adipocytes and the endocardium showed that over 95% of the intramyocardial adipocytes were within 100 \( \mu \)m from the endocardium (Figure 1E). These data showed that the intramyocardial adipocytes were highly enriched in regions close to the endocardium of the adult heart.

Lineage tracing reveals an endocardial origin of the intramyocardial adipocytes.

The above data suggested a locational correlation between intramyocardial adipocytes and the endocardium, implicating that the endocardial cells could contribute to the adipocytes. To address this hypothesis, we performed Nfatc1 promoter-driven lineage tracing experiments. Nfatc1 was specifically expressed in the endocardium of early developing heart at E9.5; however, its expression was significantly reduced at later embryonic and neonatal stages (Online Figure IIIA). Moreover, previous lineage tracing
experiments using Nfatc1-IRES-Cre knockin mouse line showed that the Nfatc1-IRES-Cre labeled endocardial cells in developing embryos.\textsuperscript{15} Therefore, we generated similar mouse line by knocking Dre recombinase into the ninth exon of Nfatc1 with a linking peptide 2A via homologous recombination (Figure 2A). Dre recombinase is a Cre-like site-specific recombinase that catalyzes recombination at the rox sites rather than the loxp sites.\textsuperscript{26, 27} We crossed Nfatc1-Dre with rox specific reporter Rosa26-rox-stop-rox-RFP, which is named as R26-RSR-RFP throughout this study.\textsuperscript{28} In Nfatc1-expressing cells, Dre-Rox recombination led to RFP labeling of Nfatc1\textsuperscript{+} cells as well as their descendants (Figure 2B). We found that Nfatc1-Dre specifically labeled endocardial cells of the developing heart by co-staining of RFP and PECAM on E9.5 heart sections (Figure 2C and 2D). Consistent with our previous work that the endocardium gives rise to coronary VECs,\textsuperscript{19} we detected that Nfatc1\textsuperscript{+} endocardial cells contribute to the majority of coronary VECs in the inner myocardium wall by co-staining PECAM or coronary VECs-specific marker FABP4\textsuperscript{29} with RFP (Online Figure IIIB and IIC).

To prove whether the labeled endocardial cells contribute to intramyocardial adipocytes, we co-stained RFP with the adipocyte marker PLIN and found that a subset of RFP\textsuperscript{+} cells also adopted the PLIN\textsuperscript{+} adipocyte fate in both left and right ventricles (Figure 2E and Online Figure IID). By performing staining of another fat tissue marker BODIPY493/503, or oil red in the consecutive heart sections, we confirmed that these intramyocardial RFP\textsuperscript{+} PLIN\textsuperscript{+} cells were fat tissues (Figure 2F,G and Online Figure IIIE,F). We also found that a subset of RFP\textsuperscript{+} cells expressed adipogenic transcription factors PPAR\text{g} and CEBP\text{a} (Online Figure IV), which were previously used to detect intramyocardial adipocytes.\textsuperscript{6} To address the percentage of intramyocardial adipocytes derived from the endocardium, we quantified PLIN\textsuperscript{+}RFP\textsuperscript{+} cells among all intramyocardial adipocytes; and found that endocardial cells contributed to 41.38 ± 5.75 % fat cells using the Dre-Rox fate mapping model. Together, these data indicate that Nfatc1\textsuperscript{+} endocardial cells contributed to intramyocardial adipocytes in adult heart.

**Inducible tracing confirms the endocardial origin of the intramyocardial adipocytes.**

One caveat in interpreting the results of genetic lineage tracing is that the expression of a reporter or fate mapping largely depends on the expression map of the Cre or Dre recombinase. Utilization of the constitutively active recombinase driven by the Nfatc1 promoter may not be able to precisely map the cell fate of endocardium due to possible activation of the recombinase at unexpected times or locations.\textsuperscript{18, 30} Therefore, the constitutively active recombinase, despite being highly efficient, might sometimes lead to over-interpretation of the results of lineage tracing studies. To address this issue, we used an inducible Cre under the control of Nfatc1 promoter (Nfatc1-CreER) and crossed it with Rosa26-loxp-stop-loxp-RFP (Rosa26-RFP) mice for mapping the cell fate of endocardial cells. Our previous study showed that this transgenic line specifically labeled the endocardial cells when tamoxifen was administered at an early embryonic stage.\textsuperscript{19} We performed tamoxifen injection at E8.5, and found specific labeling of endocardial cells at E9.5 (Online Figure VA). Although the activity of inducible Cre was not as efficient as constitutively active recombinase under the control of Nfatc1 promoter, it labeled the majority of endocardial cells (~70\%) and also their derivatives, including the coronary VECs in the inner myocardial wall.\textsuperscript{19} Furthermore, we followed the endocardial cell fate till the adult stage and found that these endocardial cells formed a substantial number of FABP4\textsuperscript{+} coronary VECs in the inner myocardial wall (Online Figure VB). In addition to coronary VECs, we found that the Nfatc1-derived cells (RFP\textsuperscript{+}) expressed the fat cell marker PLIN (Figure 3B and 3C). Quantification of RFP\textsuperscript{+} PLIN\textsuperscript{+} adipocytes showed that about 38 - 40\% intramyocardial fat cells in both right and left ventricles were derived from Nfatc1-CreER labeled endocardial cells (Figure 3D). To further confirm that the labeled RFP\textsuperscript{+} PLIN\textsuperscript{+} cells are fat tissues, we collected series of heart sections and performed both oil red staining and immunostaining for RFP and PLIN on consecutive sections. These RFP\textsuperscript{+} PLIN\textsuperscript{+} cells were also oil red\textsuperscript{+} cells (Figure 3E), verifying that these endocardium derived cells were indeed adipocytes. Immunostaining for PLIN and the brown fat-specific marker UCP1 showed that intramyocardial adipocytes were the PLIN\textsuperscript{+}UCP1\textsuperscript{−} white fat but not the PLIN\textsuperscript{+}UCP1\textsuperscript{+} brown fat (Online Figure VI). While endocardial cells contribute to intramyocardial adipocytes.
adipocytes we ask if their endothelial cell marker remained after lineage conversion. Therefore, co-staining of RFP, PLIN and endothelial cell-specific marker PECAM or endomucin (EMCN) was performed on Nfatc1-CreER;Rosa26-RFP heart sections; and we found that endocardium-derived adipocytes (RFP’PLIN’) no longer expressed endothelial cell markers PECAM or EMCN (Online Figure VII). To independently test the adipogenic potential of the endocardial cells, we isolated endocardial cells from Nfatc1-CreER;Ai47 (a GFP reporter line) and cultured in vitro for induction of adipogenesis. Oil red staining showed that these endocardial cells could differentiate into adipocytes in response to adipogenic stimuli in vitro (Online Figure VIII-A-D). We also isolated endocardial cells for single-cell culture and expansion to score for adipogenesis in vitro. Our results of oil red staining showed that clones from single endocardial cell could differentiate into adipocytes in response to adipogenic stimuli in vitro (Online Figure VIII-E,F).

To further illustrate the endocardial contribution to intramyocardial adipocytes in vivo, we administered low dosage of tamoxifen to Nfatc1-CreER;Rosa26-RFP mice and performed clonal analysis of labeled cells in vivo to focus on the fates of coronary VECs and adipocytes. We found some endocardial cells differentiated into adipocytes (type I clone), coronary VECs (type II clone), or the intermediate of both adipocytes and coronary VECs (type III clone) in the adult heart (Online Figure IX). By examining 23 hearts via immunostaining for RFP, PLIN and PECAM on serial heart sections, we detected 5 type I clones, 218 type II clones and 2 type III clones (Online Figure IX). The results from this clonal analysis suggested that most endocardial cells were unipotent and capable of differentiating into either coronary VECs or adipocytes. Only very few endocardial cells were bi-potent and capable of differentiating into both coronary VECs and adipocytes.

In the heart, endocardial cells and coronary VECs are two distinct types of endothelial cells with different morphology and molecular signatures. During both fetal and neonatal heart growth, endocardial cells contribute to a substantial number of coronary VECs in the myocardial wall. Previous study has documented that VECs in the adipose tissue are capable of giving rise to adipocytes, so we asked if the endocardium-derived coronary VECs might also trans-differentiate into intramyocardial adipocytes. To directly address this possibility, we took advantage of the molecular marker Apln that is specifically expressed by VECs but not by the endocardial cells. Our previous work also showed that Apln-CreER specifically and efficiently labeled coronary VECs but not the endocardium. We generated the Apln-CreER;Rosa26-RFP mouse line and performed triple tamoxifen treatment to label almost all coronary VECs in the developing heart (Figure 4A). By costaining RFP with the pan-endothelial cell marker PECAM, we found that Apln-CreER labeled coronary VECs (PECAM’RFP’) but not endocardial cells (PECAM’RFP-) in the neonatal P7 heart (Online FigureXA). We confirmed this result by co-staining RFP with an additional VECs marker FABP4, and found that the endocardial cells were RFP’FABP4- while almost all coronary VECs were FABP4’RFP’ (Online Figure XB). We then followed the fate of coronary VECs in adult stage when intramyocardial adipocytes have developed. Immunostaining of RFP with endothelial cell marker CDH5 on adult heart sections showed that most coronary VECs were CDH5’RFP’, while endocardial cells were RFP’CDH5’ (Figure 4B-4D), indicating that the labeling of coronary VECs in our Apln-CreER;Rosa26-RFP mouse line was efficient and specific. By immunostaining of RFP and PLIN on these sections from adult Apln-CreER;Rosa26-RFP mice, we did not detect any RFP’PLIN- cells in the right ventricle, ventricular septum or left ventricle (Figure 4E), indicating that coronary VECs did not contribute to intramyocardial adipocytes in adult heart.
**Other sources of intramyocardial adipocytes.**

To study the other possible sources of intramyocardial adipocytes, we performed cardiomyocyte-specific lineage tracing. The cardiomyocyte-specific aMHC-MerCreMer mouse line\(^3^2\) was crossed with the Rosa26-RFP reporter line to examine if labeled cardiomyocyte differentiated into intramyocardial adipocytes in the adult heart (Online Figure XIA). We treated aMHC-MerCreMer;Rosa26-RFP mice with tamoxifen at the late embryonic stage and found most cardiomyocytes were labeled in the adult heart (Online Figure XIA,B). Our results from immunostaining for RFP and PLIN showed that RFP\(^+\) cardiomyocytes did not contribute to PLIN\(^+\) intramyocardial adipocytes in the adult heart (Online Figure XIC). Neither did these labeled cardiomyocytes adopt an epicardial adipocyte cell fate in the adult heart (Online Figure XID). Therefore, it is not likely that cardiomyocytes could give rise to the intramyocardial or epicardial fat in the adult heart during homeostasis.

Nevertheless, since previous work has showed that the epicardium and its derivatives, the epicardium-derived cells (EPDCs), contribute to adipocytes during both development and post-injury \(^3^5\), we asked if EPDCs could also give rise to intramyocardial adipocytes. We crossed the epicardial Wt1-CreER line\(^3^3\) with the Rosa26-RFP reporter line to trace the cell fate of EPDCs in the adult heart. We treated Wt1-CreER;Rosa26-RFP mice with tamoxifen at an early embryonic stage (E10.5) and found efficient labeling of perivascular cells in the adult heart (Online Figure XIIA,B). Immunostaining for RFP and PLIN showed that a subset of PLIN\(^+\) intramyocardial adipocytes were RFP\(^+\) (Online Figure XIIIC), confirming that in addition to the endocardium, the epicardium also contributed to intramyocardial adipocytes during normal heart development. To examine if the endocardium, in reverse, contribute to the epicardial fat, we performed immunostaining for RFP and PLIN in the Nfatc1-CreER;Rosa26-RFP mice. We did not detect any PLIN\(^+\) epicardial adipocytes that co-expressed RFP (Online Figure XIID), indicating that the endocardium derived cells did not contribute to the epicardial fat.

**Lineage tracing of adult endocardial cells in homeostasis and after myocardial infarction.**

Our previous work has showed that adult epicardial cells could give rise to new adipocytes after myocardial infarction.\(^5\) Similarly, we asked if the adult endocardial cells still possess this adipogenic potential in homeostasis and after myocardial infarction. Since there are no inducible Cre lines for postnatal endocardial labeling, we took advantage of the inducible Cdh5-CreER line\(^3^4\) and labeled both the endocardium and coronary VECs. We treated the Cdh5-CreER;Rosa26-RFP mice with tamoxifen in the neonatal stage and examined the cell fate of RFP\(^+\) cells in the adult heart (Figure 5A). We first collected samples at week 3 post tamoxifen treatment to confirm that our labeling strategy for endothelial cells was efficient and specific. Costaining of RFP with PECAM showed that most endothelial cells including the endocardial cells were labeled as RFP\(^+\) (Figure 5B). Examination of both ventricles and the interventricular septum showed that the RFP\(^+\) cells were PECAM\(^+\) endothelial cells but not the PLIN\(^+\) intramyocardial adipocytes (Figure 5C,D). These data suggested that adult endocardial cells did not differentiate into fat cells in homeostasis.

To test if endocardium could be reactivated and differentiated into adipocytes after injury, we induced myocardial infarction (MI) by permanent ligation of left anterior descending (LAD) coronary artery. We treated the Cdh5-CreER;Rosa26-RFP mice with tamoxifen two weeks before MI, and found that most endothelial cells were labeled as RFP\(^+\) (Figure 6A,B). Four weeks after MI, large fibrotic tissue formed in the left ventricle. Moreover, we observed a few intramyocardial adipocytes that remained as RFP\(^+\) in the infarct region (Figure 6C). In both the border region of infarcted left ventricle and the remote right ventricle, we did not find any PLIN\(^+\) adipocytes that were RFP\(^+\). Unlike the adult epicardial cells, our results suggested that the adult endocardial cells did not give rise to adipocytes in homeostasis or after myocardial infarction. Nevertheless, whether adult endocardial cells generate new adipocytes in other forms of cardiovascular diseases, such as obesity-associated cardiomyopathy, requires further investigation.
DISCUSSION

In this study, we presented the endocardial origin of intramyocardial adipocytes in adult heart. Endocardium is a common origin of both coronary vascular endothelial cells and intramyocardial adipocytes. Previous study showed that vascular endothelial cells give rise to both white and brown adipocytes during development. However, our vascular endothelial cell specific lineage tracing studies showed that these intramyocardial adipocytes were not derived from coronary VECs. While most of these coronary VECs in the inner myocardial wall were derived from the endocardium, which could be a common progenitor for both coronary VECs and intramyocardial adipocytes, the endocardial cells adopt a unique developmental program for adipocyte formation (Figure 4F). It would be important to understand the molecular regulators that mediated the transition of endocardial cells to adipocytes, which requires further study in the future.

Endocardial cell is a unique type of endothelial cells that play crucial roles in many aspects of cardiovascular development. The single layer of endocardial cells lining the developing heart provides a substantial portion of mesenchyme for cardiac cushion formation through endocardial-to-mesenchymal transition. In addition to mesenchymal cells, endocardial cells also contribute to vascular endothelial cells during both embryonic development and postnatal heart growth. However, there is still controversy over the magnitude of endocardial contribution in the embryonic heart. Moreover, endocardial cells (including both endocardium and blood vessels) give rise to fibroblasts in the adult injured heart through an endocardial-to-mesenchymal transition (EMT). Recent studies suggested that most of these fibroblasts derived from EMT originate, in fact, from the endocardium during embryonic development, suggesting that endocardial cells also contribute to fibroblast formation. During cardiac injury such as myocardial infarction, endocardium is a site for endogenous arteriogenesis with a good source of endothelial cells for neovascularization. These studies suggested that adult endocardial cells could be driven to undergo EMT for a fibroblast fate or coronary vascular cell fate. Moreover, our work showed that endocardial cells could also generate adipocytes in the heart. Altogether, these studies suggest that endocardial cells could differentiate into multiple lineages during heart development, homeostasis and after injury, including coronary vascular endothelial cells, fibroblasts and adipocytes. Since it has been reported that mesenchymal cells can also be differentiated into adipocytes, it is also possible that these endocardial cells directly transdifferentiate into adipocytes, or form mesenchymal-like cells in the postnatal heart. The latter has been recently demonstrated in disease models such as endocardial fibroelastosis, in which postnatal endocardial cells undergo excessive endocardial-to-mesenchymal transition. Our work provided important evidence that endocardial cells contributed to adipocytes, but it remains elusive at present if this lineage conversion was direct or indirect. As endocardial cells give rise to mesenchymal cells and some type of mesenchymal cells have been reported to differentiate into adipocytes, it is also possible that the endocardial-to-mesenchymal transition is an intermediate stage for endocardium-to-fat transition. The differentiation of adipocytes should involve principal adipogenic factors such as PPARg and C/EBPa, which require further investigation.

It is not clear currently what functions are mediated by those intramyocardial adipocytes in normal adult heart. Previous work has suggested that adipocytes could secrete some adipocytokines, such as adiponectin and CTRP9, which have been shown to exert salutary effects on the survival of cardiomyocytes and vascular functions. Adiponectin protects against inflammation and injury in autoimmune myocarditis, reduces cardiac oxidative stress, and ameliorates cardiomyocyte hypertrophy in diabetic cardiomyopathy. CTRP9 secreted by fat cells is also cardioprotective, proangiogenic and antiapoptotic; and increased CTRP9 could restore cardiac function, attenuate cardiomyocyte apoptosis and increase neovascularization in hypoxia-induced cardiomyocyte injury. Therefore, it is likely that intramyocardial adipocytes in the normal heart exert a protective role for cardiomyocytes and coronary vessels through paracrine mechanism, which merit further investigation in the future. However, excessive adipocytes might also be associated with cardiovascular diseases, such as arrhythmogenic right ventricular cardiomyopathy. In this genetic disease,
excessive adipose tissues are found to replace myocardium of the right ventricle, which is then clinically manifested as ventricular arrhythmias and sudden cardiac death. Generation of lineage tracing tools and mouse models to address specific cardiomyopathies would enhance our understanding in the fate and function of these intramyocardial fat particularly during the adult disease states of the heart.

Unraveling the developmental origin of intramyocardial adipocytes would have implications to the understanding of pathophysiological processes of cardiovascular disease. Excessive adipocytes in the heart is one of the phenotypic hallmarks for some types of cardiomyopathy. In arrhythmogenic right ventricular cardiomyopathy, adipocytes replace cardiac myocytes in the right ventricle. Adult cardiomyocytes are terminally differentiated, which are less likely to dedifferentiate into adipocytes. Recent elegant genetic fate mapping studies using Mef2C and Nkx2.5 Cre lineage tracing models show that the adipocytes of the arrhythmogenic right ventricular cardiomyopathy are derived from the second heart field progenitor cells. Since Mef2C and Nkx2.5 Cre lines label the endocardium, vascular endothelial cells and smooth muscle in addition to the myocardium, it remains possible that other cell types (including endocardial cells or vascular endothelial cells) might give rise to adipocytes. Our study using the endocardium specific lineage tracing model suggested that some of the intramyocardial adipocytes arise from the endocardium, raising the possibility that excessive deposit of fat in the myocardium could also be of the endocardial origin in the arrhythmogenic right ventricular cardiomyopathy. In the setting of myocardial infarction, collagen has been regarded as the principal structural substrate of ventricular tachycardia. However, recent studies showed that formation of intramyocardial adipocytes in the infarcted left ventricle could contribute a significant risk factor associated with altered electrophysiological properties in ventricular tachycardia. The developmental process of epicardium-to-fat transition could be recapitulated after myocardial infarction, but the endocardium-to-fat transition during normal development was not deployed again in the injured heart. It remains unknown whether endocardial cells could give rise to excessive adipocytes in other types of cardiovascular diseases such as arrhythmogenic right ventricular cardiomyopathy or endocardial fibroelastosis, which require further studies. Indeed, additional origins, such as the second heart field progenitor cells, also provide an important source of adipocytes in arrhythmogenic right ventricular cardiomyopathy. Nevertheless, our work has demonstrated an alternative source of intramyocardial adipocytes and more research is needed to identify the pathophysiological signals that determine lineage conversion of cardiac adipocytes via endocardium-to-fat transition, paving the way for a better understanding in cardiovascular diseases including the increased incidence of obesity-associated cardiomyopathy.

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DISCLOSURES
None.
REFERENCES


FIGURE LEGENDS

**Figure 1. Intramyocardial fat is highly enriched in regions close to the endocardium of adult heart.**

A, Immunohistochemical staining of the lipid droplet-associated protein, perilipin (PLIN, brown) on embryonic and postnatal hearts at different stages. Intramyocardial PLIN+ adipocytes (arrows) start to appear at the postnatal (P) 4 week (w), P4w. B, Oil red staining on heart sections of P26w old mice. Oil-red+ fat cells are more restricted to regions close to the endocardium (arrows) and epicardium (arrowheads). B1 and B2 are magnified images showing fat cells. C, Immunostaining of PLIN and TNNI3 in P31w heart. Nuclei are stained with DAPI. Arrowheads indicate epicardial fat in the atrioventricular groove. D, Magnified image shows restricted location of PLIN+ adipocytes (arrows) in the TNNI3+ myocardium close to the endocardium. E, Quantification of the percentage of fat cells and distance away from the endocardium in adult mice hearts. 0 µm distance indicates close contact of adipocytes with the endocardium; and < 5% intramyocardial adipocytes are over 100 µm away from the endocardium. Values are shown as means ± s.e.m.; n = 3. LV, left ventricle; RV, right ventricle; VS, ventricular septum; LA, left atrium; RA, right atrium. Scale bars, 1 mm in B-C; 100 µm in A,D.

**Figure 2. Nfatc1-Dre labeled cells contribute to adipocytes.** A, Schematic diagram showing the knockin strategy of Nfatc1-Dre by homologous recombination. B, Schematic diagram showing Dre-Rox recombination for genetic lineage tracing of Nfatc1+ cells. C, Whole mount image of an E9.5 Nfatc1-Dre;R26-RSR-RFP embryo. D, Immunostaining of RFP and endothelial cell-specific marker PECAM on sections of E9.5 embryo. Arrowheads indicate RFP+PECAM+ endocardial cells. E, Immunostaining of RFP and PLIN on heart sections of P12w Nfatc1-Dre;R26-RSR-RFP mice. Arrowheads indicate RFP+PLIN+ fat cells. Asterisks indicate region encircled by endocardium. F and G, Lipid accumulation detection by BODIPY493/503 staining and oil red staining on consecutive sections from (E). Asterisks indicate region encircled by endocardium. Scale bars, 1 mm in (C), 100 µm in (D-F).

**Figure 3. Endocardial cells contribute to adipocytes in sub-endocardium of adult heart.** A, Schematic diagram showing the strategy of endocardial cell labeling and lineage tracing analysis using the Nfatc1-CreER;Rosa26-RFP mouse model at postnatal P12w to P28w. B, Immunostaining of RFP and PLIN on heart sections derived from P21w Nfatc1-CreER;Rosa26-RFP mice. Arrows indicate PLIN+ fat tissue inside heart; arrowhead points to epicardial fat in the atrioventricular groove; dotted lines demarcate epicardium (Epi). C, Magnified image of merged channels showing RFP+PLIN+ cells (arrows). D, Quantification of the percentage of genetically labeled PLIN+ cells among adipocytes in the right and left ventricle, n = 5. E, Oil red staining and immunostaining of serial heart sections derived from the Nfatc1-CreER;Rosa26-RFP mice. Oil red+ indicates fat cells. Arrows indicate co-staining of Nfatc1-derived cells (RFP+) and fat cells (PLIN+). LV, left ventricle; RV, right ventricle; Epi, epicardium; Endo, endocardium. Scale bars, 1 mm in (B); 100 µm in (C, E).

**Figure 4. Coronary VECs do not contribute to intramyocardial adipocytes.** A, Schematic diagram showing the labeling strategy of coronary VECs using the Apln-CreER;Rosa26-RFP mouse model. B, A cartoon figure showing the labeling of coronary VECs (red), but not endocardium (green) of the adult heart. C, Immunostaining of RFP, CDH5 and DAPI on heart sections from P26w adult mice. D, Interpretation of immunostaining images from (C) using pseudo colors. Most RFP(+)CDH5(+) cells are coronary VECs (red pseudo-color) and most RFP(-)CDH5(+) are endocardial cells (green pseudo-color). Almost all coronary VECs are labeled in the Apln-CreER;Rosa26-RFP mice. E, PLIN+ intramyocardial fat cells are not RFP+, suggesting that they are not derived from the RFP+ coronary VECs. F, A cartoon figure showing endocardial cells differentiate into intramyocardial adipocytes and coronary VECs. Coronary VECs do not contribute to intramyocardial adipocytes. Scale bars, 100 µm.
Figure 5. Postnatal endocardial cells do not contribute to adipocytes. A, Schematic showing strategy for tamoxifen induction at postnatal 1 week (P1w) and analysis at P12w to P36w. B, Immunostaining for RFP and PECAM on heart sections of 3 weeks old (P3w) Cdh5-CreER;Rosa26-RFP mice. Tamoxifen is injected at 2 weeks after birth. C,D, Immunostaining for RFP, PLIN and PECAM on heart sections of adult Cdh5-CreER;Rosa26-RFP mice. C1, C2 and D1 are magnified images of boxed regions in C and D respectively. RFP+ cells adopt an endothelial cell fate but not the adipocyte cell fate. LV, left ventricle; ventricular septum, VS; right ventricle, RV; Endo, endocardium. Scale bars, 100 µm. Each figure is a representative of 4 - 5 individual samples.

Figure 6. Adult endocardial cells do not contribute to intramyocardial adipocytes after myocardial infarction. A, Schematic diagram showing strategy for tamoxifen induction, myocardial infarction (MI) and analysis of the Cdh5-CreER;Rosa26-RFP lineage tracing mice. B, Immunostaining for RFP and CDH5 on heart sections before myocardial infarction (MI). C-E, Immunostaining for RFP and PLIN on heart sections of Cdh5-CreER;Rosa26-RFP adult mice. Cdh5-CreER labels both endocardial cells and vascular endothelial cells, and they do not contribute to PLIN+ adipocytes (white arrowheads). Scale bars, 100 µm. LV, left ventricle; RV, right ventricle; Endo, endocardium. Representative images are derived from three individual samples.
Novelty and Significance

What Is Known?

- Excessive cardiac fat is the phenotypic hallmark for some types of cardiomyopathy.
- Second heart field progenitor cells is a source of fat cells in arrhythmogenic right ventricular cardiomyopathy.
- Endocardium contributes to vascular endothelium.
- Vascular endothelium of the adipose tissue gives rise to fat cells.

What New Information Does This Article Contribute?

- Most intramyocardial adipocytes of the heart reside in the myocardial region close to endocardium.
- Developing endocardial cells contribute to these intramyocardial adipocytes.
- Cell fate change of endocardial cells to adipocytes does not involve an intermediate step via the vascular endothelium.
- Epicardium also contributes to intramyocardial adipocytes.

Unraveling the developmental origins of cardiac fat provides novel insights into treatment options for obesity-associated cardiovascular diseases. In this study, we found that most intramyocardial fat cells are located in the myocardial region close to endocardium. By using the genetic lineage tracing technology, we uncovered that the developing endocardium contributes to intramyocardial fat cells. The finding of endocardium-to-fat transition has important implications in the etiology and pathophysiology of the obesity-associated cardiomyopathy, such as arrhythmogenic right ventricular cardiomyopathy.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Endocardium Contributes to Cardiac Fat
Hui Zhang, Wenjuan Pu, Qiaozhen Liu, Lingjuan He, Xiuzhen Huang, Xueying Tian, Libo Zhang, Yu Nie, Shengshou Hu, Kathy Lui and Bin Zhou

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Endocardium Contributes to Cardiac Fat

Supplemental Materials

A. Detailed Materials and Methods
B. Supplemental References
C. Supplemental Figures (Online Figure I - XII)

A. Detailed Materials and Methods

Mice and tamoxifen-induced lineage tracing

All animal studies were carried out in strict accordance with the guidelines in the Institutional Animal Care and Use Committee (IACUC) of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. Mice were maintained on a C57BL6/ICR background. Tamoxifen was introduced by gavage at the indicated time (0.1-0.2mg/g). Caesarean section was performed on pregnant mice receiving tamoxifen to obtain perinatal pups. Apln-CreER, Nfatc1-CreER, Rosa26-RFP, aMHC-MerCreMer, Wt1-CreER, Cdh5-CreER mice were described previously.1-6 R26-RSR-RFP was generated from Ai66 after germline removal of loxp-stop-loxp cassette.7 Nfatc1-Dre mouse line was generated by homologous recombination using the CRISPR/Cas9 technology. The 2A peptide sequence was derived from porcine teschovirus-1 followed by the cDNA encoding Dre recombinase and polyadenylation sequence. The entire cassette was introduced into the exon 9 of Nfatc1. This mouse line was kindly generated by Shanghai Biomodel Organism. Co, Ltd. We designed PCR primers spanning the genomic DNA and inserted cassette (5’-AACGGGAAACGGAAGAGAAGC-3’, 5’-AGACATCCTCATCAGGTTGTTGAG-3’, 5’-GTGGTGTGAAGAGGCCTACAGGTATG-3’) to test the correct targeted allele with reference to the wild type allele.

Immunostaining and BIODIPY 493/503 staining

Immunostaining was performed as previously described.8 Briefly, embryos or hearts were collected in PBS, and washed with PBS to remove excessive blood. Embryos with fluorescence reporters were observed and photographed using fluorescence microscopy.
Then the embryos or hearts were fixed in 4% PFA at 4°C for 20 minutes to 1 hour. After three times washing in PBS, tissues were dehydrated in 30% sucrose over night at 4°C and embedded in OCT (Sakura). Cryosections of 8-10 μm thickness were collected. After air dry for 1 hour, slides were blocked with PBS supplemented with 0.1% trion X-100 and 5% normal donkey serum (Jackson Immuno Research) for 30 minutes at room temperature, followed by first antibody incubation at 4°C overnight. The following first antibodies were used: PLIN (Sigma, P1998), PLIN (Abcam, ab61682), RFP (Rockland, 600-401-379), RFP (ChromoTek, ABIN334653), PECAM (BD, 553370), VE-CAD or CDH5 (R&D, AF1002), FABP4 (Abcam, ab13979), TNNI3 (Abcam, ab56357). Signals were developed with Alexa fluorescence antibodies (Invitrogen). For weak signals, we used HRP-conjugated antibodies with tyramide signal amplification kit (PerkinElmer). BIODIPY 493/503 (Life Technologies, D-3922) was used at a concentration of 20 ng/ml in PBS. Cryosections were incubated with BIODIPY 493/503 for 30 minutes at room temperature and then counterstained with DAPI (Vector lab). Images were acquired by Zeiss confocal microscope (LSM510), Olympus confocal microscope (FV1200), Leica stereo-microscope (M165FC) and Olympus microscope (BX53).

**Oil Red staining**

Cryosections were air dried for 1 hour at room temperature and then rinsed in 60% isopropanol for 10 minutes. After soaked in oil red solution for 15 minutes, slides were rinsed in 60% isopropanol again until the background became transparent. Then slides were stained with Hematoxylin A for 4 minutes, followed by treatment with clarifier solution (1% concentrated hydrochloric acid in 70% ethanol) and bluing reagent (1% ammonia). After rinsing in tap water, slides were mounted in glycerin jelly. Images were acquired by Olympus microscope (BX53). For staining on cultured cells, medium was aspirated and cells were washed once in PBS. Then the cells were fixed in 4% PFA at room temperature for 15 minutes. After washing in PBS for 10 minutes, the cells were incubated with oil red solution for 30 minutes at room temperature. The cells were rinsed with 60% isopropanol and tap water once at room temperature. The red stained lipid droplets can be visualized by light microscopy. Images were obtained by Zeiss
microscope (AXIO Zoom. V16).

**In situ hybridization**

In situ hybridization was performed according to protocol described previously. Dissected embryos or hearts were fixed overnight in 4% fresh PFA and embedded in OCT (Sakura) after dehydration in 30% sucrose, which was dissolved in DEPC treated PBS. Cryosections of 8-10 μm thickness were collected. After air dry for 1 hour, the slides were incubated with anti-sense probes (1 μg/ml) overnight at 65°C. After washing in SSC and MABT buffers overnight, the slides were incubated with anti-digoxigenin antibodies for 2 hours at room temperature. Then the slides were developed with NBT and BCIP in the dark to the desired extent. The following primers were used to generate Nfatc1 probes: forward, GAGATGGAAGCAAAGACTGACCG, reverse, CAGGCTGGAGACCTCTAAAAATGC.

**Isolation of embryonic endocardial cells and adipogenic differentiation**

The Nfatc1-CreER mice were crossed with the GFP reporter line Ai47 (kindly provided by Professor Hongkui Zeng) for lineage tracing studies of endothelial cells. Tamoxifen was administered by oral gavage at E8.5 and embryonic hearts were collected at E11.5 when there were very few coronary endothelial cells in the hearts. One day before collecting the embryos, sheep anti-rat IgG Dynabeads (Invitrogen, Cat. 110.35) were prepared. 1.5 μg GFP antibody (Nacalai Tesque, Cat. 04404-84) and 25 μl Dynabeads were added into 400 μl 5% FBS diluted in PBS and incubated overnight at 4°C. On the next day, place the tube containing GFP antibody and Dynabeads were placed on a magnet (Dynal, Product No. 120.04) for 1 minute and then supernatant was then removed. After washing twice with PBS, the antibody-conjugated beads were re-suspended in 100 μl 5% FBS diluted in PBS and stored at 4°C temporarily. Then the hearts were collected from E11.5 Nfatc1-CreER;Ai47 embryos, and were cut into pieces and digested in type I collagenase (1 mg/ml) at 37°C for 45 minutes with rotation. The heart-collagenase solutions were passed through a 70 μm filter with an attempt to remove remaining the clumps of cells followed by centrifugation at 400xg for 5 minutes to discard the supernatant. The cells were re-suspended in 900 μl 5% FBS diluted in PBS and incubated
with 100 μl antibody-conjugated beads which were prepared previously for 30 minutes at 4°C with gentle rotation. The tube was then placed on a magnet for 2 minutes and the supernatant was discarded. After washing 4 times with 5% FBS diluted in PBS, the GFP+ cells which were pulled down by GFP antibody-conjugated beads were re-suspended in EGM-2 medium (Lonza, CC-3162) and plated in 1% gelatin treated culture dishes. The beads could be removed gradually after a few days in culture. To efficiently induce adipogenic differentiation, the isolated cells were incubated with TGFβ1 (Santa Cruz, Cat. Sc-146, 1ng/ml diluted in DMEM) for 15-20 days. The cells were then treated with adipogenic medium (10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM 3-Isobutyl-1-Methylxanthine, and 5 μM Rosiglitazon diluted in DMEM) or control medium for 4 days and the medium was changed every 2 days. Followed by incubation with insulin medium (10 μg/ml) or control medium for another 4 days, the cells could be ready for detecting lipid droplets. The 3T3-L1 cell lines were purchased from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Science, and cultured in 10% newborn calf serum-containing DMEM. All cells were cultured at 37°C in a humidified atmosphere consisting of 5% CO2, 95% air.

Statistics

All data were presented as mean values ± SEM. Statistical comparisons between data sets were done by a two-sided unpaired Student’s t test for comparing differences between two groups. P < 0.05 was considered to be statistically significant.

B. Supplemental References


C. Supplemental Figures (Online Figure I - XII)
Online Figure I. Location of intramyocardial and epicardial adipocytes. A, Immunostaining of lipid droplet-associated protein, PLIN (brown) on P2w to P8w hearts. Arrowheads indicate the PLIN+ fat cells in the epicardium of P3w, 4w and 8w hearts. Asterisks indicate the atrioventricular groove. A, atrium; V, ventricle; Epi, epicardium. B, Oil red staining of E15.5 to P8w heart sections. Intramyocardial fat cells were found starting at P4w. Arrows indicate oil red+ fat cells. Each image is a representative of 3 individual heart samples. Scale bars, 500 μm in (A), 100 μm in (B).
Online Figure II. Intramyocardial adipocytes are close to endocardium. A, Immunostaining for CDH5, PLIN and FABP4 on heart sections of adult wild-type mouse. PLIN+ adipocytes are in proximity with CDH5+FABP4+ endocardial cells (arrowheads). C, Immunostaining for PECAM, RFP and PLIN on Apln-CreER:Rosa26-RFP heart sections. Tamoxifen was induced at E10.5, P1, P4 to label Apln+ coronary vascular endothelial cells (RFP+). PLIN+ adipocytes are in proximity to PECAM+RFP+ endocardial cells (arrowheads). B, D, Cartoon images showing that some adipocytes (black arrowheads) are close to the innermost endocardial cells facing LV chamber, while some adipocytes (black arrows) are close to endocardial cells that reside between two blocks of myocardium. Each image is representative of 3 individual hearts. Scale bars, 100 µm.
Online Figure III. Nfatc1 expression and fate map. A, In situ hybridization of Nfatc1 on embryonic and neonatal heart sections. B,C, Immunostaining of RFP and endothelial cell-specific marker PECAM or coronary VECs-specific marker FABP4 on heart sections of adult Nfatc1-Dre;R26-RSR-RFP mice. Nfatc1-Dre labels both endocardial cells and coronary VECs. D, Immunostaining of genetic marker RFP and adipocyte marker PLIN on heart sections of 12w old Nfatc1-Dre;R26-RSR-RFP mice. Arrowheads indicate RFP+PLIN+ cells. RV, right ventricle. E,F, Images showing lipid accumulation by (E) BODIPY493/593 and (F) oil red staining on consecutive sections. Scale bars, 100 μm. Each image is a representative of 3 individual heart samples.
Online Figure IV. Nfatc1-derived cells contribute to PPARg+ or CEBPa+ adipocytes. A, Immunostaining for RFP and PPARg showed that a subset of endocardial cells contribute to PPARg+ adipocytes (arrowheads). B, Immunostaining for RFP and CEBPa showed that a subset of endocardial cells contribute to CEBPa+ adipocytes (arrowheads). Scale bars, 100 μm.
Online Figure V. The endocardium and its derivatives, coronary VECs, are labeled by Nfatc1-CreER in embryonic and postnatal hearts. A, Immunostaining of RFP and PECAM on sections of E9.5 Nfatc1-CreER;Rosa26-RFP embryos. Tamoxifen was injected at E8.5. B, Immunostaining of RFP and FABP4 on sections of P21w adult Nfatc1-CreER;Rosa26-RFP hearts. Arrows indicate RFP+FABP4+ coronary VECs. V, ventricle; a, atrium; LV, left ventricle. Scale bars, 100 µm.
Online Figure VI. Intramyocardial and epicardial adipocytes are white adipocytes. A,B, Immunostaining of PLIN and UCP1 on heart sections showed intramyocardial and epicardial adipocytes are UCP1 negative, indicating they are white adipocytes. C, As a positive control, UCP1 staining works in other brown adipocytes in peri-aortic fat tissue. Endo, endocardium; Epi, epicardium. Each image is representative figure of 3 individual samples. Scale bars, 100 μm.
Online Figure VII. Endocardium-derived adipocytes do not maintain endothelial cell markers PECAM or EMCN. A, Immunostaining for RFP, PLIN and PECAM on Nfatc1-CreER;Rosa26-RFP adult mouse hearts. Tamoxifen was injected at E8.5. Arrowheads indicate RFP+PLIN+ adipocytes that are PECAM negative. B, Immunostaining for RFP, PLIN and EMCN on heart sections shows RFP+PLIN+ adipocytes (arrowheads) are EMCN negative. Scale bars, 100 μm.
Online Figure VIII. Endocardial cells differentiate into adipocytes in vitro. A, Experimental procedures for obtaining GFP+ endocardial cells from E11.5 Nfatc1-CreER;Ai47 heart ventricles. B, Bright-field and fluorescence view of primary isolated GFP+ cells. Brown color in bright-field are Dynabeads that could be gradually removed after a few days' culture. C, Oil red staining on cultured GFP+ cells showed adipocyte formation after adipogenic medium culture (arrowheads). D, Oil red staining on 3T3-L1 cell lines with adipogenic medium as positive control for culture and staining procedures. E, Schematic figure showing strategy for expansion of single endocardial cell and adipogenic cultures. F, Oil red staining on cultured cells showed adipocyte formation after adipogenic medium culture (arrowheads). Scale bars, 100 μm.
Online Figure IX. Clonal analysis of endocardial cells. A, Schematic showing three types of endocardial cell clones: type I, only adipocyte; type II, only coronary VEC; type III, both adipocyte and coronary VEC. Tamoxifen was injected at E8.5. B, Immunostaining for RFP and PLIN on heart sections shows one clone contains endocardial cells (white arrowheads) in the endocardium (Endo) and intramyocardial fat cells (yellow arrowheads) in the inner myocardial wall (IMW). C, Immunostaining for RFP, PLIN and PECAM shows one clone contains endocardial cells (white arrowheads) and coronary VECs (red arrowheads) in the IMW. D, Immunostaining for RFP, PLIN and PECAM shows one clone contains endocardial cells, adipocytes (yellow arrowhead) and coronary VECs (red arrowhead). Scale bars, 100 μm.
Online Figure X. Coronary VECs are labeled by Apln-CreER in neonatal hearts. A, Immunostaining of RFP and pan endothelial cell marker PECAM on heart sections of P7 Apln-CreER;Rosa26-RFP mice. Tamoxifen was injected at E10.5, P1 and P4. Arrows indicate RFP+PECAM+ VECs, while arrowheads indicate RFP-PECAM+ endocardial cells. B, Immunostaining of RFP and coronary VECs marker FABP4 on heart sections of P7 Apln-CreER;Rosa26-RFP mice. Arrowheads indicate RFP+FABP4+ VECs. Endocardial cells in the innermost layer of the myocardium (arrowheads) were not labeled. RV, right ventricle; VS, ventricular septum; LV, left ventricle. Scale bars, 1 mm.
Online Figure XI. Epicardial or endocardial adipocytes are not derived from cardiomyocytes. A, Schematic showing strategy for tamoxifen induction and sample analysis. B, Immunostaining for RFP and TNNI3 on heart sections of adult aMHC-MerCreMer mice showed that most cardiomyocytes were labeled. C, Immunostaining for PLIN and RFP on heart section shows RFP+ cardiomyocytes do not contribute to PLIN+ endocardial adipocytes. D, Immunostaining for PLIN and RFP on heart sections from 17 weeks aMHC-MerCreMer mouse. RFP+ cardiomyocytes do not contribute to PLIN+ epicardial adipocytes. Asterisks indicate atrioventricular groove. LV, left ventricle; RV, right ventricle; Endo, endocardium. Scale bars, 100 µm. Each image is representative of 4 - 5 individual samples.
Online Figure XII. Study of epicardial contribution to intramyocardial adipocytes and endocardial contribution to epicardial adipocytes. 

A. Schematic showing strategy for tamoxifen induction and sample analysis. B. Immunostaining for RFP and PECAM on heart sections of adult Wt1-CreER;Rosa26-RFP mice showed efficient labeling of perivascular cells by RFP. C. Immunostaining for RFP and PLIN on heart sections of adult Wt1-CreER;Rosa26-RFP mouse. Tamoxifen was injected at E10.5. A subset of intramyocardial adipocytes are derived from Wt1+ epicardial cells (arrows), while some adipocytes (arrowheads) are not derived from Wt1+ epicardial cells. D. Immunostaining for RFP and PLIN on heart sections of adult Nfatc1-CreER;Rosa26-RFP mouse. Tamoxifen was injected at E8.5. Asterisk indicates the atrioventricular groove. No RFP+PLIN+ cells in epicardial fat tissues were detected. LV, left ventricle; LA, left atrium. Each image is a representative of three individual samples. Scale bars, 100 μm.