2011

Genetic And Physiological Contribution Of Adrenergic Cells In Heart Development

Kingsley Osuala
University of Central Florida

Part of the Medical Sciences Commons
Find similar works at: https://stars.library.ucf.edu/etd

University of Central Florida Libraries http://library.ucf.edu

This Doctoral Dissertation (Open Access) is brought to you for free and open access by STARS. It has been accepted for inclusion in Electronic Theses and Dissertations, 2004-2019 by an authorized administrator of STARS. For more information, please contact STARS@ucf.edu.

STARS Citation
https://stars.library.ucf.edu/etd/1877
GENETIC AND PHYSIOLOGICAL CONTRIBUTION OF ADRENERGIC
CELLS IN HEART DEVELOPMENT

by

KINGSLEY OSUALA
B.H.S. University of Louisville, 2002

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biomedical Sciences
in the Burnett School of Biomedical Sciences
in the College of Graduate Studies
at the University of Central Florida
Orlando, Florida

Summer Term
2011

Major Professor: Steven N. Ebert Ph.D.
ABSTRACT

The adrenergic hormones norepinephrine (NE) and epinephrine (EPI) are essential for cardiovascular development as embryos lacking NE/EPI begin to die abruptly between embryonic days 10.5 and 11.5 due to apparent cardiac failure. The objective of this research aims to elucidate the mechanism of the embryonic fatality observed in the NE/EPI deficient mouse model. Here we utilized the dopamine β-hydroxylase knockout (Dbh-/-) mouse model, which lacks the gene and subsequent enzyme necessary for the conversion of dopamine to norepinephrine. Embryonic mouse hearts were extracted from Dbh+/+ (control) and Dbh-/- (experimental model) mice for mRNA transcript expression profiling. These studies were performed using the Affymetrix Mouse Genome 430A 2.0 Arrays and quantitative real-time RT-PCR. Gene expression data suggest a novel connection between the ability of the heart to synthesize adrenergic hormones and the gene expression of enzymes involved in the synthesis of retinoic acid. Specifically, we found a statistically significant change in transcriptional expression of the retinol binding protein-1 (Rbp-1), retinol dehydrogenase 12 (Rdh-12) and beta carotene monooxygenase-1 (Bcmo-1) genes in the E10.5 Dbh-/- mouse heart. The gene expression of Rbp-1 and Rdh-12 were increased 1.4 fold and 2.1 fold on the microarray, respectively. The proteins translated from these genes play central roles in the transport and enzymatic conversion of precursor molecules in the pathway of retinoic acid biosynthesis. Additionally, we found that the expression of Bcmo-1, an enzyme responsible for the breakdown of beta carotene to the retinoic acid
precursor retinal, was down regulated 2.7 fold in the Dbh-/- heart based on microarray assessment. Bcmo-1 is a well known retinoic acid responsive gene, suggesting that the loss of adrenergic hormones in the Dbh-/- mouse heart may result in a deregulation of retinoic acid synthesis and further an alteration in the concentration of retinoic acid present in the embryonic tissue of adrenergic hormone deficient embryos.

In addition, we utilized a genetic mouse model that expresses β-galactosidase (β-Gal) in cells capable of synthesizing epinephrine in order to identify the spatial and temporal distribution of adrenergic-derived cells in the developing heart. The model was designed so that cells capable of expressing the gene phenylethanolamine N-methyltransferase (Pnmt), which is responsible for the synthesis of epinephrine, also produce the enzyme β-Gal as a reporter. The resulting presence of the β-Gal enzyme can be visualized using a β-Gal substrate called XGAL, which is converted into a blue precipitate when cleaved by the β-Gal enzyme. Evaluation of the location of these cells in the embryonic heart showed a preferential distribution at the atrioventricular sulcus at E10.5, and later at E18.5 a more widely distributed ventricular pattern was observed. In addition, the right atrium showed a cluster of XGAL positive cells (blue cells) near the region of the sinoatrial node, while the distribution of XGAL positive cells in the left atrium was quite diffuse. Interestingly, when the adult heart was examined, it was discovered that cells capable of synthesizing epinephrine (adrenergic-derived) are found predominately on the left side of the heart. This left-sided distribution appears to be non-random and non-uniform, since specific regions are consistently XGAL positive, but not every cell in each region. Whole mount and 3-dimensional reconstruction of the
β-Gal staining showed that these cells traverse the depth of the heart at the mid-ventricular and apical regions. This finding is quite interesting and may provide new knowledge about the functional and structural characteristics of the adult heart. One observation is that these cells may contribute to the cardiomyopathy known as Tako-Tsubo or “Broken Heart” syndrome. The syndrome is characterized by left ventricular dysfunction during bouts of stress. Also, of particular intrigue is the anatomical correlation of the adrenergic derived cells and the helical ventricular myocardial band (HVMB). Careful examination of the spatial and directional pattern of these cells within the myocardium suggests they contribute primarily to a specific section of the HVMB. The significance of this finding is yet to be uncovered.

Taken collectively, this study has shown a novel connection between two crucial developmental signaling pathways. Adrenergic hormone and retinoic acid signaling can now be viewed as cooperative partners in the development of the embryonic heart. In addition, this study has also shown that adrenergic derived cells in the adult heart have a distinctive left-sided distribution, which is non-random, non-uniform, and shows interesting features suggesting an anatomical connection to the HVMB and a clinical association to Tako-Tsubo syndrome. These findings will appreciably contribute to the knowledge base of the scientific community.
ACKNOWLEDGMENTS

I would like to give special thanks to my family and friends who have supported me throughout my academic career. I would also like to thank the entire Ebert lab c. 2005-2011, and my committee members; Drs. P.E. Kollattukudy, S.N. Ebert, K. Sugaya, and C. Calestani.
# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ VIII

LIST OF TABLES ............................................................................................................ XI

CHAPTER 1 ADRENERGIC HORMONES IN THE DEVELOPING HEART .................. 1
  General Introduction .................................................................................................... 1
  Adrenergic hormones and retinoic acid synthesis .................................................. 5
  Anatomical distribution of adrenergic derived cells in the mouse heart .............. 7

CHAPTER 2 ADRENERGIC HORMONES AND RETINOIC ACID: A NOVEL PAIR IN HEART DEVELOPMENT ............................................................ 10
  Introduction ............................................................................................................... 10
  Methods ..................................................................................................................... 12
    Animals ................................................................................................................... 12
    Ultrasonography .................................................................................................... 12
    Embryonic tissue collection .................................................................................. 13
    RNA Isolation ........................................................................................................ 13
    Microarray data analysis ....................................................................................... 13
    Real-time PCR analysis ......................................................................................... 14
    Retinoic acid bioassay ............................................................................................ 15
  Results ........................................................................................................................ 16
    Assessment of Adrenergic-Deficient Embryonic Heart Rates In Vivo ............... 16
    Genomic Assessment of the Adrenergic-Deficient Embryonic Heart ............... 20
    RT-PCR Validation and Assessment of Microarray Data ..................................... 26
    Retinoic Acid Concentration Assessment ............................................................ 29
  Discussion .................................................................................................................. 31

CHAPTER 3 DISTINCTIVE LEFT HEART CONTRIBUTION OF ADRENERGIC- DERIVED CELLS ................................................................. 38
  Introduction ............................................................................................................... 38
  Methods ..................................................................................................................... 41
    Materials ................................................................................................................ 41
    Mice ......................................................................................................................... 41
    Histological Preparation and Staining ................................................................. 42
    β-galactosidase activity ......................................................................................... 42
    Immunostaining .................................................................................................... 43
    2D Digital Image Analysis .................................................................................... 44
    3D Image Reconstruction ...................................................................................... 44
    Statistical Analyses ............................................................................................... 45
  Results ........................................................................................................................ 46
    Adrenergic-derived cell distribution in the adult mouse heart ......................... 46
    Quantification of XGAL+ staining in the adult mouse heart ......................... 48
LIST OF FIGURES

Figure 1. Adrenergic hormone synthesis. Synthesis pathway of the adrenergic hormones norepinephrine and epinephrine from the amino acid tyrosine. .......... 3

Figure 2. Biosynthesis of retinoic acid. Synthesis pathway of retinoic acid and its negative feedback loop with beta carotene monooxygenase. .......................... 6

Figure 3. Classical conduction system of the adult heart. The neuronal system of the heart is necessary for the propagation of electrical signals from the parasympathetic and sympathetic nervous system. ................................... 9

Figure 4. Embryonic ultrasound imaging depicts crown, heart, and rump of E10.5 embryos in live dams (A and B). Heart rates were measured at midgestation to determine embryo viability (C). Attrition rates were calculated based on detection of heart beat at the various stages of development. ....................... 18

Figure 5. Algorithm designed to identify genes having a consistent and significant change in gene expression. ................................................................. 23

Figure 6. Distribution of genes called significant on GeneLogic and S.A.M. 3.0 analyses. The data shown here is distributed by gene ontology. ................. 26

Figure 7. Validation of Microarray data. (A) RNA from the same hearts used for the microarray experiments was utilized to validate the experiment using RT-PCR. (B) The results showed a good correlation between methodologies. Of the genes probed, 5 of 8 or 62% showed similar changes in gene expression. 28

Figure 8. Microarray gene expression profile of several enzymes involved in the synthesis and metabolism of retinoic acid. A comparative analysis of Dbh-/- mouse model to adrenergic competent controls (N=4). ......................................... 30

Figure 9. Evaluation or RA concentration in embryonic tissue. A standard curve was generated to establish a detectable limit for the F9 RARE cell bioassay (A). RA concentration was measured in embryonic trunk extracts of adrenergic competent and adrenergic deficient embryos (B and C). ............. 31

Figure 10. Diagram of RA metabolism and movement in the cell via carrier proteins and enzymes.......................................................... 34

Figure 11. Vegf-A expression during midgestation is unaltered in the embryonic mouse heart. Evaluation of the gene expression of Vegf-A in the Dbh-/- mouse heart or a three day period during midgestation shows no significant difference in the level of expression vs. the Dbh+/+ heart at E9.5, E10.5, or E11.5 (N=3, 3, and 6).......................................................... 35

Figure 12. Adult mouse heart showing the distribution of Pnmt expressing cells. 2D slices show how PNMT expressing cells penetrate the depth of specific regions of the left ventricle (LV), and nearly fill the left atria (LA) (A-H). Whole mount staining shows a 3 dimensional surface view of the PNMT expressing cells (I-K). .......................................................... 48

Figure 13. To quantify Pnmt expression, Pnmt^{+/-Cre}, ROSA26^{+/-Bgal} adult mouse heart images were partitioned into right and left heart. The line of separation originates from the apex and extends toward the base of the heart and through the aorta (bold line). Additional partitioning isolates the IVS from
THE RVFW AND LVFW (PANEL B AND D). EACH PARTITION WAS THEN ANALYZED TO DETERMINE THE TOTAL PIXELS CONTAINING THE BLUE XGAL+ STAIN. THE LEFT HEART SHOWS HEAVY XGAL+ STAINING WHEN COMPARED TO THE RIGHT SIDE OF THE HEART. P_{NMT}^{+/+}, ROSA26^{Bgal/Bgal} CONTROL HEART SHOWS NO XGAL+ STAINING (PANEL C AND D). ................................................................. 50

FIGURE 14. THREE-DIMENSIONAL RECONSTRUCTION OF THE XGAL+ STAINING IN THE P_{NMT}^{+/Cre}, ROSA26^{Bgal/Bgal} HEART. (A) REPRESENTATIVE TWO-DIMENSIONAL SECTION OF THE ADULT MOUSE HEART WERE CUT AT 20 MICRONS. (B) STILL-SHOT OF A 3D-RENDERED IMAGE GENERATED FROM STACKED 2D IMAGES AS DESCRIBED IN THE METHODS SECTION. THE ARROWS POINT TO EQUIVALENT REGIONS IN BOTH PANELS THAT ROUGHLY CORRESPOND TO BASAL, MID, AND APICAL CONCENTRATIONS OF XGAL+ CELLS IN THESE HEARTS. PLEASE SEE ONLINE SUPPLEMENTAL FIGURES 1 AND 2 FOR DIFFERENT PERSPECTIVE VIEWS OF THE 3D IMAGE ANALYSIS FOR THESE DATA. ................................. 52

FIGURE 15. P_{NMT}-CRE EXPRESSION IN CARDIOMYOCYTES OF THE ADULT MOUSE HEART. ..... 53

FIGURE 16. HIGH-MAGNIFICATION PHASE-CONTRAST AND LIGHT MICROSCOPY IMAGES OF XGAL-STAINED CELLS IN THE LV FREE WALL FROM AN ADULT MOUSE HEART. (A) BLACK AND WHITE PHASE-CONTRAST IMAGE OF XGAL-STAINED MYOCYTES (ARROWS). (B) COLOR LIGHT MICROSCOPY IMAGE OF AN XGAL-STAINED MYOCYTE (ARROW). INVERSE IMAGES OF (A) AND (B) ARE SHOWN IN PANELS (C) AND (D), RESPECTIVELY. NOTE THE CHARACTERISTIC STRIATED RECTANGULAR-LIKE SHAPE OF THE STAINED CELLS... 55


FIGURE 18. ACTIVE P_{NMT} EXPRESSION IN THE ADULT MOUSE ADRENAL GLANDS AND HEART. THE ADRENAL GLAND AND HEART OF ADULT P_{NMT}^{+/Cre}, ROSA26^{+/Bgal} MICE WERE EXTRACTED TO EVALUATE THE DISTRIBUTION OF ACTIVE P_{NMT} EXPRESSION. CRYOSECTIONS OF THE MOUSE ADRENAL GLAND WERE IMMUNOFLOUORESCENTLY STAINED TO IDENTIFY P_{NMT} EXPRESSION (GREEN). THE ADRENAL MEDULLA (MED) SHOWED BRIGHT POSITIVE STAINING AS EXPECTED, WHILE THE CORTEX (CTX) OF THE ADRENAL GLAND SHOWED ONLY BACKGROUND FLUORESCENCE (A). HIGH MAGNIFICATION IMAGES OF P_{NMT} IMMUNOFLOUORESCENT STAINING IN THE LV REVEALS SMALL CELLS POSITIVE FOR CYTOPLASMIC P_{NMT} EXPRESSION IN THE HEART (B, ARROWS) AND ADRENAL MEDULLA (C). NEGATIVE CONTROL FOR THE LV IMAGE SHOWN IN PANEL B ILLUSTRATES THE ABSENCE OF FLUORESCENT SIGNAL IN THE RED SPECTRUM (D). ......................................................... 58

FIGURE 19. P_{NMT} IS EXPRESSED IN ALL CHAMBERS OF THE ADULT MOUSE HEART. IMMUNOFLOUORESCENT STAINING IN THE ADULT MOUSE HEART USING AN ANTI-P_{NMT} ANTIBODY SHOWS ACTIVE EXPRESSION IN BOTH RIGHT AND LEFT ATRIA (A-C). THE MAJORITY OF FLUORESCENTLY LABELED CELLS (GREEN) IN THE ATRIA HAVE A TRIANGULAR-SHAPED APPEARANCE (ARROWS) WITH NEURONAL-LIKE EXTENSIONS (ASTERISKS). IN
ADDITION, CARDIOMYOCYTE-LIKE CELLS ALSO SHOW POSITIVE Pmnt staining (arrowheads) which appears to be cytoplasmic while a few cells show staining along their lateral border (d-f). The ventricular myocardium also displayed small triangular-shaped cells which were positive for Pmnt expression (e, arrows). Figure 20. High-magnification confocal co-immunofluorescent staining for Pmnt and sarcomeric α-actinin in adult mouse heart (LV) sections. Pmnt immunofluorescent staining in the adult mouse heart identifies small triangular-shaped cells (green) (A, B arrows) within the interstitial spaces. Cardiomyocyte-specific staining for sarcomeric α-actinin (red) shows identifiable striations in branching cells. Immunofluorescent co-staining with Pmnt and sarcomeric α-actinin shows apparent Pmnt expression within some cardiomyocytes (C, arrowheads).
LIST OF TABLES

Table 1. Oligomer sequences utilized for RT-PCR reactions including; GenBank® accession ID, predicted product size, and annealing temperatures. .................. 15

Table 2. Genes determined to be significantly altered on microarray by Gene Logic analysis (P ≤ 0.05). ........................................................................................................... 21

Table 3. List of 21 genes determined to be significantly altered after the S.A.M. 3.0 analysis. .................................................................................................................. 24

Table 4. Quantification of XGAL+ staining in the adult mouse heart after right/left demarcation......................................................................................... 49
CHAPTER 1 ADRENERGIC HORMONES IN THE DEVELOPING HEART

General Introduction

Heart disease is the leading cause of death in the United States. It has been declared an epidemic by several institutions including the Center for Disease Control, National Institute of Health and the American Heart Association. Each institute has enhanced their research efforts to uncover the cultural, socioeconomic, and molecular basis of heart disease. In the U.S. alone, approximately 40% of the total annual deaths are attributed to some type of heart disease [1]. This translates to more than 600,000 people who have died from heart disease in the past 12 months. Sadly, infants account for an unmerited percentage of those deaths. Congenital heart defects are estimated to arise in 28,000 births and will result in an additional 25,000 embryonic fatalities this year in the United States [2]. For these reasons, researchers are working diligently to understand the complexity of heart disease, and to develop strategies to battle the mortality rate this terrible condition encompasses.

Studies of the molecular mechanisms underlying heart failure have led to many new and interesting discoveries. In the past decade, protein expression studies have revealed that heart disease has a proteomic profile, much like that observed in many cancers. Particular proteins of interest include; C-reactive protein (CRP), monocyte chemo-attractant protein-1 (MCP-1), and insulin-like growth factor binding protein [3] [4] [5]. These among others have been directly associated with known degenerative
mechanisms of heart failure and are prime targets for future clinical therapeutics. The goal of the current study is to append such novel molecular findings and further our understanding of the principal mechanisms of cardiac development and disease.

Adrenergic hormones have long been associated with physiological reactions, including the well known reactions associated with our flight or fight response. There are two classical adrenergic hormones; norepinephrine (NE) and epinephrine (EPI). The adrenergic hormones NE and EPI act as neurotransmitters in the nervous system and as hormones in the endocrine system. Together, NE and EPI facilitate many physiologic functions of the human body.

The principal locations of adrenergic hormone synthesis are the medulla of the adrenal glands, and the postganglionic terminals of the nervous system [6]. Hormonal activity of NE and EPI is mediated through secretion directly into the blood stream where these hormones bind the alpha adrenergic receptors (α1, α2), and/or the beta adrenergic receptors (β1, β2, and β3). Receptor binding results in downstream signaling events such as; the activation of protein kinases A and C, calcium storage release in muscle cells, and the activation of phosphorylases which initiate the breakdown glycogen [7] [8]. In this manner, adrenergic hormones play a major role in the body’s homeostasis control. Some of the physiological changes include but are not limited to; increased heart rate, vasodilatation, vasoconstriction, sweating, increased respiration, iris dilation and decreased movement within the GI tract [9].

The enzymes responsible for the synthesis of adrenergic hormones are tyrosine hydroxylase (TH), dopa decarboxylase (DDC), dopamine β-hydroxylase (DBH), and
phenylethanolamine N-methyltransferase (PNMT) in their respective order of epinephrine generation from the precursor molecule tyrosine (Figure 1). Three of these enzymes have been genetically disrupted in the mouse model, which has giving rise to three different phenotypic profiles. The Th knockout (Th-/-) mouse model is deficient of dopamine (DA) and the downstream end products NE and EPI. The Th-/- phenotype is embryonic lethal with 75-100 % fatality in utero due to apparent cardiovascular failure beginning at embryonic days 11.5 [10].

Figure 1. Adrenergic hormone synthesis. Synthesis pathway of the adrenergic hormones norepinephrine and epinephrine from the amino acid tyrosine.
Interestingly, the Dbh knockout (Dbh-/-) mice, which produce the monoamine DA but not NE or EPI, show a slightly more severe phenotype than the Th-/- mouse model, as they begin to die one day earlier and less than 50% of the embryos survive to embryonic day 15.5, with a limited occurrence of survival to birth [11]. Conversely, the Pnmt-/- mice do not appear to have a salient phenotype, as they survive to birth and appear normal [12]. These findings indicate a critical necessity for NE availability during embryonic development.

Detailed studies of the Dbh-/- mouse model have shown that NE is critical for embryonic survival beyond embryonic day 10.5, since half of the Dbh-/- mice die between E10.5 and E11.5. This would represent a period equivalent to 4 - 5 weeks in human fetal development, and is the time immediately following the initial onset of the embryonic heart beat [13]. The reason for the observed embryonic fatality in the Dbh-/- mouse model has been attributed to cardiac failure, although, of an unknown etiology due to the lack of detectable structural abnormalities in the heart. Detailed analyses have shown that the embryonic heart spontaneous begins to fail near mid-gestation, exhibiting arrhythmogenesis plus cardiac and hepatic pooling of the blood [11]. It has been suggested that the NE/EPI deficient mouse heart may fail due to severe hypoxia and an inability to recover from hypoxic events in utero [14] [15]. This is a possible mechanism of embryonic fatality because it is well established that adrenergic hormones are released under hypoxic conditions to stimulate downstream effectors responsible for survival during hypoxic stress [16].
This study examined the underlying physiologic and genetic mechanisms involved in the embryonic cardiac failure resulting from the genetic deletion of the Dbh gene. In addition, fate-mapping strategies were used to investigate the localization of cells capable of synthesizing adrenergic hormones in the developing mouse heart.

Adrenergic hormones and retinoic acid synthesis

Recently, genetic profiling of the Dbh-/- mouse heart has implicated the retinoic acid signaling pathway as a participant in the embryonic lethal phenotype. Retinoic acid is essential for normal embryonic development and plays a critical role in cellular differentiation and gene transcription during early development. It has been shown to play a central role in eye development, vision, CNS development, immune system maintenance and specifically cardiac development [17] [18] [19] [20]. Vitamin A (retinol) is one of the two primary dietary precursors of retinoic acid. The synthesis of retinoic acid from vitamin A or retinol is achieved by the retinol dehydrogenase (RDH) family of enzymes, which convert retinol to retinal via oxyreductase activity [21]. The other retinoic acid precursor is beta carotene (β-carotene), which is enzymatic cleaved via the enzyme beta carotene-15, 15 mono-oxygenase-1 (BCMO-1) to yield two identical molecules of retinal [22]. The resulting retinal molecules can then be oxidized to retinoic acid by the aldehyde dehydrogenase 2 (ALDH1A2) enzyme, formerly retinaldehyde dehydrogenase 2 (RALDH2) [23], which is the rate limiting step in the synthesis of retinoic acid in the embryonic mouse heart [18] (Figure 2).
During embryonic development, the location and concentration of retinoic acid is stringently modulated by its synthesizing and degradation enzymes to facilitate normal development [17][24]. Disrupting the embryonic homeostasis of retinoic acid concentration results in several abnormalities. Studies have shown that administration of retinoic acid to embryonic tissue during embryogenesis leads to many disorders including; craniofacial malformations, disruption of central nervous system (CNS) development, cardiac development alterations, teratogenesis, and embryonic lethality [25, 26]. Equally, a retinoic acid deficiency results in many similar developmental abnormalities. These include; a disruption in A-P patterning of the CNS, abnormal liver development, early hematopoeisis, and cardiac progenitor cell defects [27,28].
The role of retinoic acid during these early stages of development is primarily regulation of gene expression. The function of retinoic acid is mediated through the retinoic acid receptors (RAR α, β, γ) and the retinoid X receptors (RXR α, β, γ) [26] [29]. These nuclear receptors form homozygous and heterozygous dimers with one another and other nuclear receptor which bind to DNA at retinoic acid response elements (RARE) or other response elements to regulate specific gene activation or suppression [30,31]. In this manner, retinoic acid can act as an inducer or suppressor of gene expression in a concentration and temporal dependent way [31, 32]. This reiterates the necessity for stringent regulation of retinoic acid synthesis during embryonic development.

Anatomical distribution of adrenergic derived cells in the mouse heart

The anatomical structure of the heart plays an important role in its function. The phrase 'Form predicts Function' has become central dogma in the land of biomedical research. The developing heart is subject to, and a prime example of this principle. The heart is a simple collection of cardiomyocytes, connective tissue, and neurons. Yet, the structure and complex assembly of these cells make the heart an efficient pump. Research into the architecture of the heart has shown that the cellular orientation of cardiomyocytes in conjunction with fibrous connective tissue form functional structural units called “laminas” [33]. These laminas, through autonomous action supplement the action of surrounding laminae to generate an orchestrated directional contraction of the heart resulting in an efficient and directed propulsion of blood though the heart [34]. Francisco Torrent-Guasp unraveled the macro structure of
the adult heart in a detailed stepwise manner which disassembled the myocardium based on the natural direction and assembly of these laminar units. When fully dismantled, the heart resembles a band much like that in which it originally began its development. Dr. Guasp called it the “helical ventricular myocardial band” (HVMB) [35].

The cardiomyocytes of the heart have long been thought to function primarily as mechanical units. However, studies have shown that basal levels of serum norepinephrine remain steady after complete adrenalectomy, suggesting an alternative source of systemic hormones [36]. The heart makes an idea candidate for an extra-adrenal source of adrenergic hormones as it serves as the core of the circulatory system. In 1996, Huang et al. introduced the term intrinsic cardiac adrenergic (ICA) cells, for the sub population of cells in the heart capable of producing adrenergic hormones [37]. These cells were found initially to be present in the embryonic heart at E15.5 and were primarily associated with regions of the pace-making and conduction tissue. These “ICA cells” appeared as early as embryonic day 8.5 (E8.5) in mice, and are suspected to remain in the developing heart through neonatal stages of development [12]. If these ICA cells are capable of producing the adrenergic hormones NE and EPI, it is then feasible that they may also have the capability to secrete them. So, the heart could in fact, be the source of norepinephrine found in the serum of adrenalectomized animals.

This study determined whether cells capable of producing adrenergic hormones remained in the heart beyond the embryonic stages of development. And whether ICA
cells are co-localized with common structures of the neuronal system, such as the SA node, AV node, or the bundle branches of the adult heart (figure 3).

Figure 3. Classical conduction system of the adult heart. The neuronal system of the heart is necessary for the propagation of electrical signals from the parasympathetic and sympathetic nervous system.
Introduction

Adrenergic hormones play an essential yet unexplained role in mammalian heart development during embryogenesis [10, 11]. In adult mammals, the primary adrenergic hormones are norepinephrine (NE) and epinephrine (EPI), which serve as both neurotransmitters and hormones. In the periphery, they are mainly produced in sympathetic neurons and adrenal chromaffin cells, respectively [38], though they have also been found to lesser extent in a number of other cells and tissues [39, 40]. They are secreted during periods of stress where they bind and activate various $\alpha$- and $\beta$-adrenergic receptor subtypes leading to a myriad of physiological changes associated with stress responses. In the mid 1990’s, it was discovered that NE and/or EPI are vital for embryonic survival in utero since disruption of the genes encoding for the catecholamine biosynthetic enzymes, tyrosine hydroxylase (Th) or dopamine $\beta$- hydroxylase (Dbh), resulted in an embryonic lethality due to apparent heart failure [10, 11]. In the absence of NE and EPI, embryos survive and appear relatively “normal” until approximately embryonic day 10.5 (E10.5), but then begin to die over the next several days with ~90% of the Dbh-/- mice not surviving to birth [11].

Examination of Th-/- and Dbh-/- embryos indicated that many of them had blood congestion in the liver and major vessels, suggesting poor circulation [10]. In addition, thinning of the atrial chamber walls and some disorganization of ventricular myocytes
were also observed in these mutant embryos. Consequently, the heart appeared to harbor relatively subtle morphological defects in the absence of adrenergic hormones, but impairment and loss of function appear to be the primary reasons for the observed demise of these adrenergic-deficient embryos.

Subsequent studies have shown suggested that adrenergic-deficient embryos have an impaired ability to respond to intermittent hypoxia in utero [14, 15]. Adrenergic-competent controls typically respond to hypoxia with adrenergic-mediated increases in heart rate, but Th/-/- mice cannot do this since they lack the ability to produce adrenergic hormones. Consequently, this lack of responsiveness has been hypothesized to lead to heart failure and death in Th/-/- embryos [15]. This hypothesis is supported by additional data showing that a number of known hypoxia-induced genes were significantly up-regulated in E12.5 Th/-/- embryos compared to littermate controls in response to hypoxic conditions [15]. It is not entirely clear, however, that the developing embryo/fetus is subjected to significant hypoxia during the course of a normal pregnancy. Further, the bulk of the hypoxia-related experiments were performed between E11.5 to birth, yet approximately 50% of adrenergic-deficient embryos die between E10.5 and E11.5. Thus, there are some open questions about how catecholamines influence cardiac function particularly at early stages of embryonic heart development.

We previously showed that disruption of the EPI-specific biosynthetic enzyme, phenylethanolamine n-methyltransferase (Pnmt), results in a loss of EPI but do not display any obvious developmental phenotype. Thus, NE appears to be the primary catecholamine needed for embryonic survival. In the present study, we utilized the
Dbh−/− mouse model to evaluate physiological and genetic changes associated with the loss of NE and EPI during a critical early period of embryonic heart development extending from E9.5-E12.5. We employed a combination of non-invasive high-resolution ultrasonic imaging and genome-wide microarray analyses to evaluate cardiac changes that occurred due to the absence of endogenous adrenergic hormones. The results of these experiments suggest that mechanisms unrelated to or preceding hypoxia-driven effects likely play an important role in mediating adrenergic signals during critical early phases of embryonic heart development.

Methods

Animals

All procedures and care of the mice were conducted in accordance with National Institutes of Health guidelines using protocols established by the Institutional Guidelines for the Care and Use of Animals in Research. The genetic mouse model was developed as previously described [11].

Ultrasonography

High resolution ultrasound imaging (Visualsonics Vevo 660 with 40 and 55 MHz transducers) was used to measure physiologic heart rates from Dbh−/− and Dbh+/+ embryos in vivo.
Embryonic tissue collection

For microarray studies, Dbh+/− sire and dam were mated generating Dbh+/+, Dbh+/−, and Dbh−/− offspring in Mendelian ratios. For RA measurements, Dbh+/− sire were mated with L-dihydroxyphenylserine rescued Dbh−/− dams to yield Dbh+/− and Dbh−/− offspring at a predicted 50:50 ratio as previously described [11]. Timed pregnancies yielded E9.5, E10.5, and E11.5 embryos which were used for these studies. The embryos were examined for viability and the heart was visualized microscopically for beating activity. Embryos with beating hearts were utilized for the experiments described here. Embryonic hearts were extracted and immediately frozen in liquid nitrogen unless used for ex vivo drug treated cultures. Embryonic hearts used for ex vivo cultures were excised and rinsed in PBS then placed in pre dosed cardiac differentiation media for 24 h culture. Genotype determination was carried out using genomic DNA extracted from a fraction of the embryonic tissue.

RNA Isolation

Hearts collected for mRNA expression analysis were flash frozen in liquid nitrogen. Frozen embryonic hearts were homogenized via sonication followed by Trizol® RNA extraction, Sigma Aldrich (St. Louis, MO). Total RNA quality and yield was assessed using the NanoDrop™ 1000. Only total RNA samples showing an OD260/OD280 ratio higher than 1.75 were used for microarray and real-time PCR.

Microarray data analysis

Isolated RNA from frozen embryonic hearts was sent to AffyMetrix® (Santa Clara, CA) for total genome analysis on the Mouse Genome 430A 2.0 Array. The data
output was processed by Gene Logic (Gaithersburg, MD) and sent to our laboratory for further analysis. We designed a data filtration algorithm which would generate a list of genes that showed high probability of transcript presence and a statistically significant change of mean signal between Dbh-/- and Dbh+/- samples.

Real-time PCR analysis

Total RNA samples showing an OD260/OD280 ratio higher than 1.75 were used for cDNA synthesis. Genomic DNA was removed from the isolated RNA by digestion with RNase-free DNase (Promega, Madison, WI). The cDNA was prepared using Affinity Script™ QPCR cDNA synthesis kit (600559, Stratagene, Cedar Creek, TX). We used 0.4 μg total RNA for each cDNA synthesis reaction. Real-time PCR reactions were performed using IQ SYBR Green super mix (Bio-Rad Hercules, CA) and run in the Bio-Rad iCycler. Oligonucleotide sequences and annealing temperatures are shown in table 1.
Table 1. Oligomer sequences utilized for RT-PCR reactions including; GenBank® accession ID, predicted product size, and annealing temperatures.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>GenBank ID</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurofilamentin 1 (1)</td>
<td>NM_010897</td>
<td>F: 5'GCATTGGCCAATCATGTAATGT3'</td>
<td>356</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'TCTGGTAAAGTTAAGGGCTGAGCAG3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurofilamentin 1 (2)</td>
<td>NM_010897</td>
<td>F: 5'CCGAGACGACAAGCCCTGAGC3'</td>
<td>53</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'TCTGGTAAAGTTAAGGGCTGAGCAG3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol Binding Protein 1</td>
<td>NM_011254</td>
<td>F: 5'GCATTCTGTCAGTGACAGAT3'</td>
<td>188</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'ACCAACAAAGCCAAGGAT3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>NM_021335</td>
<td>F: 5'GCACCTTGGAGAGGACTACT3'</td>
<td>134</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'AGGCCCTCCAAAGCATTTG3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF 2</td>
<td>NM_006010</td>
<td>F: 5'ATGGCTCTCTTCAGGAAAGGCTG3'</td>
<td>200</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'GCGAGCGCAAGCTGAGATCCAGG3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH 1-A2</td>
<td>NM_009022</td>
<td>F: 5'GCTTGAGCATCACAGCATG3'</td>
<td>210</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'CACTGTCTCTCTGAGATAACG3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>NM_021486</td>
<td>SUPERARRAY.PFME2381A</td>
<td>130</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lf Receptor</td>
<td>NM_013584</td>
<td>F: 5'GATCTCTAAACACAGAGAGAGG3'</td>
<td>240</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'TCAGAGACAGATGCTGACAC3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZFP-191</td>
<td>NM_021559</td>
<td>F: 5'CCAAGTAAATGAGCTACCTG3'</td>
<td>220</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'TCTAAATACGTCATCTGACG3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>XM_00479322</td>
<td>F: 5'AGAGATGATGACCCCTTGGC3'</td>
<td>200</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'CCATCACCAGTTCCAGAGAGG3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF-A</td>
<td>NM_00105250</td>
<td>F: 5'CGACAGAAAGGAGAGCACAGAGTCCC3'</td>
<td>256</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'TG0CTTGGTGAGGTTGATCCG3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Retinoic acid bioassay

F9-RARE-LacZ cells were maintained in L-15 Leibovitz media containing 20% heat inactivated, charcoal stripped Fetal Bovine Serum (FBS) and 1% PenStrep (V/V).
The cells were grown to confluency (80-90%) on 100 mm gelatin-coated dishes then plated at 60% confluency on 24 well dishes and allowed to settle overnight. Immediately before the assay individual embryonic trunks were homogenized via 5 s sonication twice in F9-RARE-LacZ cell culture assay media (passage media plus 1.6 g/L D-glucose) under dim lighting. The homogenate was centrifuged at 8000g for 5 m to removed cell debris before adding to the F9-RARE-LacZ cells. Before adding the homogenate, the normal passage media was removed from the F9-RARE-LacZ cells and 0.9 ml of assay media was added followed by 100 µl of the embryonic trunk homogenate. The co-culture was allowed to incubate overnight at 37°C and 5% CO₂.

For the detection of beta galactosidase activity using the fluorescein di-β-D-galactopyranoside (FDG) substrate; co-cultured F9-RARE-lacZ cells were lysed in 100µl of Z-buffer (0.8 g Na₂HPO₄, 0.28 g NaH₂PO₄, 0.5 ml 1M KCl, 0.05 ml 1M MgSO₄ and 0.135 ml β-mercaptoethanol, all dissolved in dH₂O to a volume of 50 ml) via 5 s sonication and added to a 96 well plate with the addition of 2 µl of 1 mM FDG. The light production was measured using the EnVision® Multilabel plate reader (PerkinElmer, USA).

Results

Assessment of Adrenergic-Deficient Embryonic Heart Rates In Vivo

To gain insight into how adrenergic deficiency leads to embryonic heart failure and death in utero, we used high-resolution ultrasound imaging to evaluate cardiac performance in adrenergic-deficient (Dbh-/-) and adrenergic-competent (Dbh+/+ and
Dbh+/-) embryonic mouse hearts in vivo. Example images from this analysis are shown in Fig. 4 where sagittal views of an E10.5 mouse illustrate the ability to detect cardiac contractions and measure crown-rump length as an estimation of embryonic age. Heart rates were assessed on E9.5, E10.5, and E11.5. Embryos were then collected for genotyping and genomic expression analyses as described in the Materials and Methods section. No significant differences were observed in mean heart rates from adrenergic-deficient mouse embryos compared to controls at any of these ages (Fig. 4 C).
Figure 4. Embryonic ultrasound imaging depicts crown, heart, and rump of E10.5 embryos in live dams (A and B). Heart rates were measured at midgestation to determine embryo viability (C). Attrition rates were calculated based on detection of heart beat at the various stages of development.

It is important to note, however, that we only measured heart rates from embryos that were alive, and thus did not factor in zeros for those hearts not beating at the time of isolation. Overall attrition rates were similar between genotypes at E9.5 and E10.5, but increased dramatically in the Dbh−/− group at E11.5, with roughly half of the
adrenergic-deficient population not surviving by this stage of development (Fig. 4D). These findings are consistent with the original reports about these mice showing that most Dbh-/ mice survive through E10.5, but then steadily decline in numbers over the next few days with >80% dead by E15.5 in heterozygous (Dbh+/-) dams [11]. It appears that the Dbh-/ embryos have nearly indistinguishable heart rates compared with their adrenergic-competent counterparts in utero until shortly before their demise. Since we recorded heart rate information from the same sets of embryos on successive developmental days, we were able to witness this phenomenon directly. In some cases, heart rates would appear essentially normal for all genotypes at E9.5, E10.5, and E11.5, but occasionally we would see some of the hearts that were beating well at E9.5, E10.5, or even E11.5, begin to develop slow, labored contractions sometimes with associated asynchronous and arrhythmic activity. In other cases, the slow labored irregular contractile activity first appeared at E11.5 or E12.5, typically followed by death within 24 hrs. Consequently, it appears that beating rates develop normally during the early phase of heart development, but then start to decline beginning around E10.5 in some Dbh-/ hearts but not in others. There appears to be a window that extends for a few days where the heart is vulnerable to the absence of adrenergic hormones, but this does not always become evident until several days after E10.5.

We took advantage of this observation by using the echocardiographic assessments to identify viable hearts of various genotypes (Dbh+/+, Dbh+-, and Dbh-/-) that were beating at normal physiological rates. The hearts were then collected immediately following echocardiography for analysis of gene expression differences
between adrenergic-competent and adrenergic-deficient embryonic mouse hearts at E10.5, near the beginning of the developmental period where embryos show greatest vulnerability to adrenergic deficiency [10, 11]. The objective of this experiment was to determine if there were underlying alterations in gene expression profiles that precede the physiological decline and ultimate death of Dbh-/- embryos in utero. The larger goal of this study was to determine how adrenergic signaling plays an essential role during a critical period of embryonic heart development. To minimize confounding variables related to downstream gene expression changes incurred as a consequence of heart failure in general, we sought to obtain early specimens that were asymptomatic. The criteria that we used included assessment of heart rates, crown-rump lengths, and general morphological/structural appearance at time of dissection. The embryos that we collected for this analysis were indistinguishable from each other in terms of these criteria and all appeared to be in relatively good health at the time of isolation at E10.5.

Genomic Assessment of the Adrenergic-Deficient Embryonic Heart

We thus identified four viable Dbh-/- experimental and four Dbh+/+ control age- and size-matched E10.5 mouse hearts for the microarray analysis. To assess changes in gene expression between these two groups, we extracted RNA and analyzed the samples using Mouse Genome 430A 2.0 Arrays (Affymetrix®, Santa Clara, CA). These arrays permit simultaneous evaluation of > 22,000 annotated genes in the mouse genome. The microarray data were initially analyzed using the Affymetrix® MAS5.0 software. This yielded a list of 22 genes that were significantly (p < 0.05) altered by a factor of two or more between E10.5 Dbh-/- and Dbh+/+ hearts (Table 2).
Table 2. Genes determined to be significantly altered on microarray by Gene Logic analysis (P ≤ 0.05).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Gene Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcno1</td>
<td>monooxygenase</td>
<td>metabolism</td>
<td>-2.7</td>
</tr>
<tr>
<td>Pla2g2d</td>
<td>phospholipase A2, group IID</td>
<td>lipid catabolism</td>
<td>-2.7</td>
</tr>
<tr>
<td>Ptgr1</td>
<td>prostaglandin F receptor</td>
<td>signal transduction</td>
<td>-2.6</td>
</tr>
<tr>
<td>Otub1</td>
<td>OTU domain, ubiquitin aldehyde binding 1</td>
<td>ubiquitin cycle</td>
<td>-2.4</td>
</tr>
<tr>
<td>Cacna2d2</td>
<td>calcium channel, voltage-dependent, alpha 2/delta subunit 2</td>
<td>calcium ion transport</td>
<td>-2.3</td>
</tr>
<tr>
<td>Nelf</td>
<td>nasal embryonic LHRH factor</td>
<td>unknown</td>
<td>-2.2</td>
</tr>
<tr>
<td>Nf1</td>
<td>neurofibromatosis 1</td>
<td>regulation of glial cell differentiation</td>
<td>-2.2</td>
</tr>
<tr>
<td>Pcdhac2</td>
<td>protocaderhin alpha subfamily C, 2</td>
<td>cell adhesion</td>
<td>-2.1</td>
</tr>
<tr>
<td>Jak3</td>
<td>insulin-like 3</td>
<td>protein amino acid phosphorylation</td>
<td>-2.1</td>
</tr>
<tr>
<td>Rdh12</td>
<td>retinoic acid dehydrogenase 12</td>
<td>visual perception</td>
<td>2.1</td>
</tr>
<tr>
<td>Ndufb2</td>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2</td>
<td>metabolism</td>
<td>2.1</td>
</tr>
<tr>
<td>Cdk6rap2</td>
<td>CDK5 regulatory subunit associated protein 2</td>
<td>biological process</td>
<td>2.1</td>
</tr>
<tr>
<td>Gaint2</td>
<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalectosaminyltransferase 2</td>
<td>protein amino acid O-linked glycosylation</td>
<td>2.1</td>
</tr>
<tr>
<td>Fmod</td>
<td>fibromodulin</td>
<td>ECM</td>
<td>2.2</td>
</tr>
<tr>
<td>Pqlc2</td>
<td>PQ loop repeat containing 2</td>
<td>signal transduction</td>
<td>2.3</td>
</tr>
<tr>
<td>Mgp</td>
<td>matrix Gla protein</td>
<td>bone mineralization</td>
<td>2.4</td>
</tr>
<tr>
<td>Col9a3</td>
<td>procollagen, type IX, alpha 3</td>
<td>cell adhesion</td>
<td>2.5</td>
</tr>
<tr>
<td>Fmod</td>
<td>fibromodulin</td>
<td>ECM</td>
<td>2.7</td>
</tr>
<tr>
<td>Ebf2</td>
<td>early B-cell factor 2</td>
<td>transcription</td>
<td>2.7</td>
</tr>
<tr>
<td>Kif2c</td>
<td>kinesin family member 2C</td>
<td>microtubule-based movement</td>
<td>3.1</td>
</tr>
<tr>
<td>Kcnj6</td>
<td>potassium inwardly-rectifying channel, subfamily J, member 6</td>
<td>transport</td>
<td>3.4</td>
</tr>
<tr>
<td>C1q3</td>
<td>C1q-like 3</td>
<td>phosphate transport</td>
<td>3.9</td>
</tr>
<tr>
<td>Fgf20</td>
<td>fibroblast growth factor 20</td>
<td>signal transduction</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Nine of these genes were down-regulated in Dbh-/- hearts relative to Dbh+/+ hearts, with the retinoic acid biosynthetic enzyme, beta-carotene 15,15'-
monooxygenase (Bcmo1) and phospholipase A2, group IID (Pla2g2d) genes displaying the greatest decreases (-2.7-fold). Thirteen genes showed strong increased expression, with fibroblast growth factor 20 (Fgf20) commanding the greatest increase (+5.3-fold). One gene, fibromodulin (Fmod), was identified twice (from two different target sequences on the array) as a gene displaying increased expression (+2.2-fold and +2.7-fold) in the adrenergic-deficient hearts.

Secondary analysis of the entire data set using a filtration algorithm generated a list of 114 genes that showed high probability of mRNA transcript presence and a statistically significant (p ≤ 0.05) change of mean expression between Dbh-/- and Dbh+/+ samples. A flow-chart outline of the criteria used for this analysis is provided in figure 5.
Figure 5. Algorithm designed to identify genes having a consistent and significant change in gene expression.
Table 3. List of 21 genes determined to be significantly altered after the S.A.M. 3.0 analysis.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Gene Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lifr</td>
<td>leukemia inhibitory factor receptor</td>
<td>signal transduction</td>
<td>-2.0</td>
</tr>
<tr>
<td>Adamts5</td>
<td>Adamts5</td>
<td>proteolysis</td>
<td>-1.9</td>
</tr>
<tr>
<td>Sf3b5</td>
<td>splicing factor 3b, subunit 5</td>
<td>nuclear mRNA splicing</td>
<td>-1.6</td>
</tr>
<tr>
<td>Cmtrm6</td>
<td>CKLF-like MARVEL transmembrane 6</td>
<td>signal transduction</td>
<td>-1.5</td>
</tr>
<tr>
<td>Shank3</td>
<td>SH3/ankyrin domain gene 3</td>
<td>intracellular signaling</td>
<td>-1.5</td>
</tr>
<tr>
<td>E030031F02Rik</td>
<td>RIKEN cDNA E030031F02 gene</td>
<td>process unknown</td>
<td>-1.5</td>
</tr>
<tr>
<td>Ppp3cc</td>
<td>protein phosphatase 3</td>
<td>intracellular signaling</td>
<td>-1.5</td>
</tr>
<tr>
<td>Nkiras1</td>
<td>NFkB inhibitor interacting Ras-like protein 1</td>
<td>protein transport</td>
<td>-1.4</td>
</tr>
<tr>
<td>Zfp191</td>
<td>zinc finger protein 191</td>
<td>transcription</td>
<td>-1.4</td>
</tr>
<tr>
<td>Rps6</td>
<td>ribosomal protein S6</td>
<td>ribosome biogenesis</td>
<td>-1.4</td>
</tr>
<tr>
<td>Pik3c2a</td>
<td>phosphatidylinositol 3-kinase, C2 alpha</td>
<td>intracellular signaling</td>
<td>-1.4</td>
</tr>
<tr>
<td>Gna1</td>
<td>guanine binding protein a inhibiting 1</td>
<td>Receptor signaling</td>
<td>-1.4</td>
</tr>
<tr>
<td>Spfh1</td>
<td>SPFH domain family, member 1</td>
<td>process unknown</td>
<td>-1.4</td>
</tr>
<tr>
<td>Gla</td>
<td>galactosidase, alpha</td>
<td>carbohydrate metabolism</td>
<td>-1.3</td>
</tr>
<tr>
<td>Ctn</td>
<td>cortactin</td>
<td>actin nucleation</td>
<td>-1.3</td>
</tr>
<tr>
<td>Zim1</td>
<td>zinc finger, imprinted 1</td>
<td>transcription</td>
<td>-1.3</td>
</tr>
<tr>
<td>Acvr2a</td>
<td>activin receptor IIA</td>
<td>signal transduction</td>
<td>-1.3</td>
</tr>
<tr>
<td>Rnd3</td>
<td>Rho family GTPase 3</td>
<td>signal transduction</td>
<td>-1.3</td>
</tr>
<tr>
<td>Pign</td>
<td>phosphatidylinositol glycan, class N</td>
<td>Gpi-anchor biosynthesis</td>
<td>-1.3</td>
</tr>
<tr>
<td>BC046331</td>
<td>cDNA sequence BC046331</td>
<td>process unknown</td>
<td>1.5</td>
</tr>
<tr>
<td>Fmod</td>
<td>fibromodulin</td>
<td>extracellular matrix</td>
<td>1.7</td>
</tr>
</tbody>
</table>

This method generated a list of genes that showed a lower fold-change (≤ 2-fold) than that found in the MAS 5.0 analysis, but showed greater consistency in signal detection among each of the 4 samples within the Dbh-/- and Dbh+/- groups. These genes were then analyzed using the publicly available significance of microarray analysis 3.0 (SAM 3.0) method [41]. This methodology generated a list of 21 significantly altered genes (Table 3).
Only one of the genes identified by the SAM 3.0 analysis was found in common with the initial MAS 5.0 analysis, and that was Fmod, which displayed a 1.7-fold increase in Dbh-/− hearts relative to Dbh+/+ control hearts. As with many genes, Fmod is represented multiple times on the array, and three of the six Fmod target sequences on these arrays were identified as showing significantly elevated expression levels in the Dbh-/− hearts when analyzed by either the MAS 5.0 or SAM 3.0 methods. From the SAM analysis, Fmod expression showed the greatest enhancement in Dbh-/− hearts relative to Dbh+/+ hearts, while the leukemia inhibitory factor receptor (Lifr) showed the greatest decline (-2.0-fold) (Table 3).

Many interesting and unexpected gene expression changes were found from both of the analysis methods employed here. Before proceeding to analysis of individual genes, we first looked at the types of changes that occurred as a result of the absence of adrenergic hormones in these E10.5 mouse hearts by evaluating the distribution of broad categories of gene expression changes observed from the MAS 5.0 and SAM 3.0 methods. As shown in the pie-charts depicted in figure 6, genes involved in metabolism and signal transduction combined for over half the total number of significant changes observed using either method. Other categories of genes showing significant differences were those involved in transcription (e.g., Ebf2, Zfp191, and Zim1), transport (e.g., Cacna2d2, Kcnj6, C1qI3, and Nkiras1), and extracellular matrix or cell structure (e.g., Fmod, Pcdhac2, Mgp, Cttm, and Pign), as well as several genes of unknown function.
Figure 6. Distribution of genes called significant on GeneLogic and S.A.M. 3.0 analyses. The data shown here is distributed by gene ontology.

RT-PCR Validation and Assessment of Microarray Data

To verify expression changes observed from the microarray analyses, we selected specific candidate genes from those listed in Tables 2 and 3 for quantitative mRNA analysis using real-time PCR (RT-PCR). Because Fmod was consistently found to be elevated by both microarray data analyses, this was one of the targets chosen. Another gene that appeared to be a good candidate for further study was the neurofibromatosis 1 (Nf1) gene because targeted disruption of this gene has been shown to lead to embryonic lethality due to heart failure during a similar stage of embryonic development in mice [42]. Although there does not appear to be another single gene from the microarray analyses (Tables 2 and 3) that has been identified to cause similar embryonic lethality when knocked out or over-expressed, there were several genes found to be important in the synthesis of retinoic acid, notably including Bcmo1 [43] and Retinol dehydrogenase 12 (Rdh12) [21]. In addition, we also included
the aldehyde dehydrogenase 1A2 (Aldh1A2, also known as Retinaldehyde dehydrogenase 2, Raldh2) gene, because this is an essential retinoic acid biosynthetic gene during early heart development [44]. The Retinol-binding protein 1 (Rbp1) was also included in the analysis since expression of this gene is often used as a surrogate for evaluating retinoic acid concentrations because its expression levels typically reflect changes in retinoic acid concentrations in the tissue being examined [45].

Fmod expression appeared similar when evaluated either by microarray or RT-PCR. In contrast, there was not good correlation between the microarray and RT-PCR results for Nf1. We tested two different sets of primer sequences specific for the Nf1 gene, but neither showed much similarity to the microarray results for this gene. Bcmo1, on the other hand, displayed strong down-regulation in Dbh-/- hearts regardless of whether it was analyzed using the microarray or RT-PCR results (Figure 7). This experiment was repeated several times with independent mRNA samples, and we always obtained similar results indicating strong down-regulation of this gene in E10.5 Dbh-/- mouse hearts relative to Dbh+/+ control hearts. The Aldh1A2 and Rbp1 genes each displayed nearly 2-fold increased expression in Dbh-/- hearts from the microarray data, and both were corroborated by the RT-PCR data to varying degrees. In the case of Aldh1A2, only a relatively slight increase in expression was observed from the RT-PCR results, whereas Rbp1 displayed comparatively more robust expression when measured by RT-PCR (Figure 7).
Figure 7. Validation of Microarray data. (A) RNA from the same hearts used for the microarray experiments was utilized to validate the experiment using RT-PCR. (B) The results showed a good correlation between methodologies. Of the genes probed, 5 of 8 or 62% showed similar changes in gene expression.
Retinoic Acid Concentration Assessment

These results indicated that retinoic acid synthesis may be perturbed in adrenergic-deficient E10.5 mouse hearts, but it was not initially clear if this was true because one biosynthetic enzyme gene, Bcmo1, was strongly down-regulated in the absence of adrenergic hormones while expression of the primary retinoic acid biosynthetic enzyme gene, Aldh1A2, appeared to be up-regulated. The increases in Rbp1 expression also indicated that RA may be increased in adrenergic-deficient E10.5 hearts.

Consequently, we decided to examine expression of several other genes thought to play important roles in RA synthesis, transport, and metabolism. The expression profiles for several of these genes are shown in Fig. 8. From these data, it is clear that only Bcmo1 is down-regulated in the absence of endogenous adrenergic hormones (Dbh-/-), while most of the rest of the RA-related genes analyzed here showed increased expression in E10.5 Dbh-/- mouse hearts relative to control Dbh+/+ hearts. Earlier studies have shown that Bcmo1 expression is down-regulated in response to elevated tissue RA concentrations, suggesting a possible feedback inhibition mechanism for expression of Bcmo1 [22].
Figure 8. Microarray gene expression profile of several enzymes involved in the synthesis and metabolism of retinoic acid. A comparative analysis of Dbh−/− mouse model to adrenergic competent controls (n=4).

To determine if tissue RA concentrations were actually altered in adrenergic-deficient embryos, we employed a sensitive bio-assay using the previously described F9-RARE-LacZ cell line [46, 47]. This cell line produces the β-galactosidase (βgal) reporter gene under the control of a promoter that is exquisitely sensitive to RA concentrations, and hence, βgal output is directly proportional to the concentration of RA present. Thus, a standard curve was generated using known concentrations of RA (Figure 9A) thereby enabling measurement of RA in extracts from individual E10.5 mouse embryos applied directly to the cultured cells. The results show that RA concentrations were significantly (p < 0.05) elevated by an average of > 3-fold in adrenergic-deficient hearts compared to adrenergic-competent controls (Figure 9 B and C).
Figure 9. Evaluation of RA concentration in embryonic tissue. A standard curve was generated to establish a detectable limit for the F9 RARE cell bioassay (A). RA concentration was measured in embryonic trunk extracts of adrenergic competent and adrenergic deficient embryos (B and C).

Discussion

The importance of adrenergic hormones in the embryonic mouse heart is evident as mice lacking the ability to synthesize NE and EPI begin to die in utero at E10.5, and very few survive to E13.5 [11]. To elucidate the mechanisms which lead to the embryonic cardiac failure of the Dbh-/- mice, we collected E10.5 hearts from Dbh-/- embryos which showed no obvious signs of cardiac distress. The hearts were first analyzed using high resolution ultrasound, to access viability and heart rate. We then
selected only those mice with comparable heart rates to Dbh+/+ mice for microarray analysis of gene expression.

The microarray analysis was predominately a two part analysis. A standard basic analysis and a more detailed analysis which took into account mathematical skewing. The basic analysis was conducted by Gene Logic which separated out genes showing a 2-fold average change in expression and a student T-test significance of \( p \leq 0.05 \). The result of this analysis yielded 23 altered genes, which is less than 1% of the total genes assayed on the microarray chip. These highlighted genes belonged to many different categories of cellular function, such as; signal transduction, metabolism, extra cellular matrix components, gene transcription and protein transport. To highlight genes having a consistent and significant differential expression across each sample set, we developed an algorithm that would generate a list of genes that were expressed above the background noise of the assay and consisted of signal values which could be compared to one another equivalently. When we compared the results of the S.A.M. 3.0 analysis to that of the MAS5.0 analysis, we found that only fibromodulin showed up on both list. However, many of the genes on the S.A.M. 3.0 list were associated with the same pathways seen in the MAS5.0 analysis. Both list of differentially expressed genes revealed interesting facts about the gene expression profile of the Dbh-/- mouse heart at E10.5. Collectively, the data suggest genetic alterations of genes involved in signal transduction and metabolism of the Dbh-/- mouse heart. The altered genes representing these two ontologies remarkably account for 52 % of the aberrantly
expressed genes in the Dbh-/- mouse heart. This observation is consistent in both the MAS5.0 and S.A.M. 3.0 analysis of the microarray data.

When we independently examined genes within associated functional pathways using real-time RT-PCR, we found changes in gene expression that were consistent with known pathway associations between those genes. For example, the retinoic acid synthesis genes Aldh1A2, Bcmo-1, and Rbp-1, showed gene expression changes consistent with a change in cellular retinoic acid concentration (Figure 10) [48-50]. Others have shown that Bcmo-1 and Rbp-1 have an inverse gene expression relationship, which we observed in our data [17, 51]. We showed here that retinoic acid concentration and/or signaling in the Dbh-/- mouse heart is affected as a result. This also suggests that the adrenergic hormones regulate retinoic acid homeostasis during embryonic mouse heart development.
It has been suggested that the loss of adrenergic hormones results in an inability embryos to respond and survive the chronic hypoxia experienced during development [14]. It has been shown that embryonic hypoxia induces the release of norepinephrine [52], which is known to lead to vasodilatation and increased blood flow to overcome cellular hypoxia. However, the Dbh-/- myocardium cannot release NE and would therefore endure fatal degrees of hypoxia. It is well known that Vegf-A and Hif1α are robustly expressed under conditions of hypoxia [53], and would be differentially expressed in the Dbh-/- mouse heart if the heart were failing due to hypoxia. Our data show that the gene expression of Vegf-A and Hif1α in the Dbh-/- heart are not
significantly different from the Dbh+/+ controls (Figure 11). Portbury et al. 2003, suggests that NE is essential for acute reversal of bradycardia due to hypoxic conditions during embryonic development and that the hypoxic conditions can be eliminated thru oxygen homeostasis [15, 54]. However, our in vivo data indicate that the loss of adrenergic signaling, specifically NE, does not lead to hypoxic conditions. The lack of significant change in the expression of these key hypoxia inducible genes indicates that the Dbh-/ mouse model may not be susceptible to hypoxic injury due to the loss of adrenergic hormones. But, suggest that the Dbh-/ mouse heart does not suffer from chronic hypoxia anymore than the Dbh+/+ littermates.

Figure 11. Vegf-A expression during midgestation is unaltered in the embryonic mouse heart. Evaluation of the gene expression of Vegf-A in the Dbh-/ mouse heart or a three day period during midgestation shows no significant difference in the level of expression vs. the Dbh+/+ heart at E9.5, E10.5, or E11.5 (n=3, 3, and 6).
The gene expression changes observed in the Dbh-/- mouse hearts reveal gene ontology’s in five different categories. Interestingly, 52% of the altered genes fall into two categories; signal transduction and cellular metabolism. We found that Rdh-12, a short chain dehydrogenase responsible for the oxidation and/or reduction of all-trans retinoids had up-regulated gene expression in the Dbh-/- mouse heart. Rdh-12 can convert retinol to retinal, or reduce retinal to retinol [21]. These metabolites are the precursor of retinoic acid; therefore, this could indicate that the Dbh-/- mouse may have an altered synthesis of retinol or retinal leading to altered retinoic acid synthesis [55] [56].

Another differentially expressed gene involved with retinol availability is Rbp-1. Rbp-1 is expressed widely in the developing embryo and plays a role in sequestration and delivery of retinol to cells for conversion to retinoic acid or storage [57, 58]. The Dbh-/- mouse heart shows a 2 fold increase in the expression of Rbp-1 determined by real-time RT-PCR. This finding also suggest an excess of cellular retinoic acid since Rbp-1 has been shown to be a direct target of retinoic acid mediated gene expression [45].

Bcmo-1 is responsible for the conversion of beta carotene to retinal in the biosynthesis pathway of retinoic acid [59]. Bcmo-1 was significantly down regulated in the Dbh-/- mouse heart. This finding suggests either a down-regulation by retinoic acid or an insufficient activation of Bcmo-1 gene expression. Studies have shown that Bcmo-1 is a direct target of retinoic acid in a negative feedback system [51, 60]. These
findings along with other supportive data indicate an increase in the concentration of retinoic acid in the Dbh-/- mouse model.

When we examined the concentration of retinoic acid in the Dbh-/- mouse heart, we found a significantly higher concentration in the Dbh-/- mouse hearts as compared to the Dbh+/+ controls. This indicates that the E10.5 Dbh-/- mouse heart does not maintain retinoic acid homeostasis at E10.5 like their Dbh+/+ counterparts. This is a major finding since it has been well documented that an excess of retinoic acid during embryonic development can lead mid-gestational embryonic lethality [61-63].
CHAPTER 3 DISTINCTIVE LEFT HEART CONTRIBUTION OF ADRENERGIC-DERIVED CELLS

Introduction

Cardiac development is a multifarious process that involves cell specification, determination, patterning, migration, and growth. This is achieved through precise regulation of gene expression within several cell types during embryonic development. The production of signaling molecules designed to coordinate these events during embryonic development is crucial. Among the multitude of signaling molecules are the adrenergic hormones, adrenaline and noradrenaline. Molecular studies of the function of adrenergic hormones during embryonic development have shown that they are vital for cardiac development because mice lacking adrenergic hormones die in utero near midgestation due to apparent heart failure [11].

Adrenergic cells are distributed throughout the developing heart, including regions associated with pacemaking and conduction tissue [64, 65]. In mice, cardiac adrenergic cells appear as early as embryonic day 8.5 (E8.5), and remain in the developing heart through neonatal stages of development [12]. These cells are responsible for the production and secretion of adrenaline and noradrenaline [64-66]. Immunohistochemical staining experiments for adrenergic biosynthetic enzymes, including tyrosine hydroxylase (Th), dopamine β-hydroxylase (Dbh), and phenylethanolamine n-methyltransferase (Pnmt), indicated transient expression of an
adrenergic cellular phenotype in different regions of the heart as development proceeds [37, 65].

Most of the anatomical work with adrenergic cells in the heart has focused on adrenal chromaffin-like cells that are commonly found in “glomus-like clusters” in and around cardiac ganglia from a wide variety of species [67-73]. More recent work suggests that adrenergic cells are also found in a subset cardiomyocytes [12, 66, 74]. Indeed, Pnmt enzymatic activity and adrenaline are found in both cardiac atria and ventricles from rats [75-79], mice [78], and humans [80-82]. Further, Pnmt activity increases dramatically following chemical denervation with 6-hydroxydopamine (6-OHDA), indicating that it is likely expressed in non-neuronal tissue within the heart [83, 84]. The precise anatomical localization of Pnmt-expressing cells within the cardiac atria and ventricles has not been described previously.

In the present study, we explore the distribution of adrenergic-derived cells within the adult mouse heart. To accomplish this objective, we used the previously established Pnmt-Cre mouse model, in which the Cre-recombinase gene was knocked-in to the Pnmt gene locus. When crossing Pnmt-Cre mice with ROSA 26 reporter (R26R) mice [85], the β-galactosidase (βGAL) reporter gene was exclusively activated in adrenergic (Pnmt-expressing) cells [12, 86]. It is important to note that cells marked by βGAL expression in this model reflect distribution of cells that are derived from an adrenergic lineage even if they no longer actively express Pnmt and the other adrenergic enzymes [66, 74]. We utilized standard histological staining techniques
combined with computer-aided three-dimensional reconstruction to provide a detailed anatomical characterization of the fate of adrenergic cells in the adult mouse heart.
Methods

Materials

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) except where noted otherwise.

Mice

The Pnmt<sup>+</sup>/Cre<sup>−</sup> and ROSA26<sup>+</sup>/βgal<sup>−</sup> (R26R) mice have been described previously [12]. The Pnmt<sup>+</sup>/Cre<sup>−</sup> mice were derived after several backcrosses (4-6) with wild-type 129/SvJ mice (stock # 0006910; Jackson Laboratories, Bar Harbor, ME), and the R26R line was backcrossed with wild-type C57Bl/6 mice (stock #000664; Jackson Laboratories, Bar Harbor, ME) for a similar number of generations (4-6). Each strain was then independently maintained in homozygous condition (Pnmt<sup>Cre/Cre</sup> and ROSA26<sup>βgal/βgal</sup>, respectively) in the Transgenic Animal Facility at the University of Central Florida where they were housed on a 12:12h light:dark cycle and provided with food and water ad libitum. For all experiments described here, Pnmt<sup>Cre/Cre</sup> and ROSA26<sup>βgal/βgal</sup> mice were mated, and the resulting heterozygous offspring were studied. All animal experiments were conducted in accordance with the University of Central Florida Animal Care and Use Committee, consistent with regulations for vertebrate animal research outlined by the National Institutes of Health (NIH). The protocol (#08-32) was approved by the University of Central Florida Animal Care and Use Committee on August 18, 2010. This approval has been renewed annually since
the initial approval date of August 24, 2008. The title of the protocol is “Molecular imaging of novel cardiomyocyte stem cells”, and it is active through August 17, 2011.

Histological Preparation and Staining

Adult (8-10 weeks of age) Pnmt^{+/Cre} ROSA26^{+/βgal} mice were anesthetized with 2.5% isoflurane and sacrificed by decapitation while under full anesthesia. The heart was rapidly removed and perfused retrogradely via a cannula positioned in the aorta. After flushing with phosphate-buffered saline (PBS), the heart was fixed by gentle perfusion with 20 mls of 2% paraformaldehyde in PBS followed by immersion in the same solution for an additional 24h at 4°C. The hearts were then transferred to a solution of 30% sucrose containing 0.02% sodium azide in PBS for at least another 24h, until ready for cryostat sectioning. The hearts were then embedded in Tissue-Tek® freezing gel (EM Sciences, Hatfield, PA) for sectioning using a Microm HM 505 N cryostat set to a temperature of -26°C to -28°C. The hearts were cut at 14-20 microns and mounted onto Super-Frost Plus microscope slides (Fisher Scientific, Inc., Pittsburgh, PA). Tissue sections were stained immediately or stored at 4°C for subsequent use.

β-galactosidase activity

Tissue sections were stained for β-galactosidase activity using 50mM XGAL dissolved in dimethylformamide as described previously [12]. XGAL was then diluted in XGAL staining buffer solution (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 1mM MgCl2, and .01% Tween-20 in PBS) at a dilution factor of 1:39. Slides were rinsed in PBS for 10 mins and incubated in the diluted XGAL solution
overnight at 37°C. The following day, the slides were washed in PBS 3 times for 10 mins each. Sections were counterstained with eosin followed by ethanol dehydration and clearing in xylene for 10 mins before adding a coverslip using Permount (Fisher Scientific, Inc., Pittsburgh, PA). Low-magnification digital images of histology sections were obtained using a Leica MZ16A stereomicroscope and Leica DFC 320 camera system. Higher magnification images were acquired using an upright Nikon Eclipse E600 light microscope with attached SPOT RTTM Slider camera (Diagnostics Instruments, Inc., Sterling Heights, MI).

Immunostaining

For immunofluorescent staining experiments, the tissue was prepared as above, and stored at -20°C until use. The general staining procedure was performed as previously described [3], with some modifications as indicated in the following text. The slides were then thawed, heat-dried at 37°C for 2 minutes prior to permeabilization in 0.1 N HCL at room temperature for 5 minutes followed by a single wash with tap water. The sections were then incubated in PBS containing 10% Tween 20 for 6 hours followed by two 15-min washes in PBS without Tween 20. Blocking was performed by incubating in PBS containing 5% fetal bovine serum (Hyclone Labs; Logan, UT) for 30 minutes prior to incubation for 2 hours at 37°C in a humidified chamber with a rabbit anti-Pnmt primary antibody (AB110) from Millipore, Inc. (Temecula, CA) at a dilution of 1:250 in PBS. In some experiments co-staining with a mouse anti-sarcomeric α-actinin (A7811, Sigma-Aldrich; St. Louis, MO) at a dilution of 1:100 in PBS was also performed. The sections were then washed twice with PBS alone for 15-min each, followed by 30-
min incubation with a FITC-conjugated donkey anti-rabbit IgG (Jackson Immunolabs, Bar Harbor, ME) and/or an Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen, Inc.; Carlsbad, CA). The sections were again washed with PBS as above, then dried and cover-slipped for fluorescent microscopy.

2D Digital Image Analysis

Images were analyzed using Adobe Photoshop™ software. For βGAL quantification, the size of the pictures was adjusted to 669300 pixels (690 x 970 pixels) per image. This adjustment was confirmed with the image histogram option in the Photoshop software. Once all images were set to the equivalent size, we used the Photoshop “magic wand” to select all blue staining from the left, and then the right side of the heart. Histogram analysis of the βGAL staining on the left and the right side of the heart yielded a total number of blue pixels for each side of the heart. The average pixel value for each individual heart was derived from analysis of 5-10 random sections.

3D Image Reconstruction

For a series of 2D slices of the stained heart, we applied a color-based clustering technique to segment regions of interest (i.e., XGAL-stained regions) and then reconstructed the corresponding 3D volume. Among all of the 2D slices, a subset of slices of interest can be manually selected according to different experiential goals. The segmentation for each selected slice went through three steps. First, a slice was converted into the L*a*b* color space. Next, the k-means algorithm was employed using squared Euclidean distance to cluster the color feature of the slice into several clusters, where each cluster corresponds to a different color range. Note that the number of
clusters, \( K \), is experimentally determined for each slice in view of the intensity inhomogeneity among the slices. While \( K \) is initialized for each slice by 6, it can be interactively tuned (increasing or decreasing) in order to find the optimal segment that corresponds to the XGAL-stained regions the best. Then, the optimal segment is extracted according to the labeling index of the corresponding cluster. Repeating the above steps to each slice, we obtained respective XGAL-stained regions for all of the slices of interest. Subsequently, a rigid registration (i.e., translation and rotation) are applied to align the segments obtained from different slices. After that, the aligned segments are sequentially stacked for 3D volume reconstruction. Note that in order to simulate the thickness of a slice, the segmented XGAL-stained regions of each slice are repeatedly stacked for 7 times. The stacked 3D data are saved in MetaImage format (.mhd). Finally, we utilized a free tool called ITK-SNAP (http://www.itksnap.org/pmwiki/pmwiki.php) to load the 3D data and construct the 3D shape of the XGAL-stained volume. ITK-SNAP provided 2D views from axial, sagittal, and coronal directions as well as 3D visualization of the volume that can be observed from dynamic viewpoints.

Statistical Analyses

All data are presented as the Mean ± S.E.M. Statistical significance was evaluated using the Student’s T-test, with \( P < 0.05 \) required to reject the null hypothesis.
Results

To identify adrenergic-derived cells in the adult mouse heart, we first set up matings between Pnmt<sup>Cre/Cre</sup>, ROSA26<sup>+/+</sup> and Pnmt<sup>+/+</sup>, ROSA26<sup>βgal/βgal</sup> as previously described [12]. The offspring from these matings were allowed to develop normally until adulthood (8-10 weeks), at which time they were sacrificed, and the hearts collected for histological staining analyses. In this model, cells that express βgal are indicative of cells that are derived from an adrenergic lineage due to their expression of Cre-recombinase from the Pnmt genetic locus. βgal expression is readily visualized as blue cells following XGAL histological staining. Examples of this staining are shown in a series of representative adult heart sections in figure 12.

Adrenergic-derived cell distribution in the adult mouse heart

XGAL+ cells were observed in several regions of the myocardium. For example, some of the most strongly positive XGAL staining was consistently observed in the left atrium (LA); thereby indicating that many of the myocytes in the LA were derived from an adrenergic lineage. The left ventricle (LV) was also stained heavily with XGAL in an apparently non-random and non-uniform pattern. In sections from the ventral surface of the heart, we saw an area of strong XGAL+ staining near the apex (Figure 12a), but as we progressed from ventral towards more dorsal sections, the LV staining pattern appeared to shift first to a more medial location along the longitudinal axis of the heart (Figure 12, panels b and c), and then continued to shift towards the base of the heart (Figure 12, panels d-f). In addition, the XGAL staining pattern seemed to generally redistribute from the periphery towards a more interior position when moving from
ventral to dorsal locations (Figure 12, panels b-f). XGAL staining of the papillary muscle (PM) was primarily localized to the superior border, with minimal staining at the right base of the muscle (Figure 12, panels b and c). XGAL+ staining of the PM was unchanged as the sections were analyzed from ventral to dorsal views. No XGAL+ staining was observed in endothelial cells lining the aorta, pulmonary trunk, or cardiac vessels.

The interventricular septum (IVS) showed intermediate levels of XGAL+ staining with a preferential tendency for localization in the right side of the septum (Figure 12, panels b-f). This pattern of staining was observed throughout the entire depth of the heart. As seen in figure 12, the XGAL+ staining in the body of the IVS was sparse throughout the heart however, we observed a small concentrated cluster of XGAL+ cells along the basal crest of the IVS (Figure 12, panels d-f). The right ventricular free wall (RVFW) had minimal XGAL+ staining in most sections of the adult mouse heart (Figure 12, panels a-f). Notably, however, XGAL+ staining in the RVFW was localized to two small areas, one near the apex and another near the base (Figure 12, panels g and h). Additionally, whole mount staining of the adult mouse heart reveals the same unique pattern (Figure 12, panels i-k). These results were consistent in each of the adult mouse hearts analyzed, thus suggesting that while adrenergic-derived cells are primarily localized on the left, there are smaller contingencies of these cells in the IVS and RV.
Quantification of XGAL+ staining in the adult mouse heart

To quantify the XGAL+ staining of the adult mouse heart, we analyzed the total XGAL+ cells in both atria and both free walls of the ventricles. To do this, we first
demarcated the major sections of the heart and created a right/left line of separation (Figure 13). We then quantified blue pixels in images of random sections from each heart. Multiple sections were analyzed from three different hearts, and the results are summarized in Table 4. The combined results show that on average, 89% of XGAL+ cells were found on the left side of the heart. As a negative control, we quantified XGAL+ staining in hearts resulting from Pnmt+/+ROSA26βgal/βgal x Pnmt+/+ROSA26+/+ matings (Figure 13, panels c and d). As expected, no XGAL+ staining was observed in these control hearts. These results demonstrate that XGAL+ staining was predominantly, though not exclusively, present in the atria and ventricular chambers of the left myocardium in the adult mouse.

Table 4. Quantification of XGAL+ staining in the adult mouse heart after right/left demarcation.

<table>
<thead>
<tr>
<th>Heart</th>
<th>Mean β-Gal positive pixels per five random slices</th>
<th>% β-Gal positive pixels per five random slices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>R26R-control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R26R-Cre-1</td>
<td>7031 ± 1306</td>
<td>965 ± 412</td>
</tr>
<tr>
<td>R26R-Cre-2</td>
<td>22806 ± 2588</td>
<td>1827 ± 79</td>
</tr>
<tr>
<td>R26R-Cre-3</td>
<td>37763 ± 14518</td>
<td>2521 ± 304</td>
</tr>
<tr>
<td>Mean, R26R-Cre (n=3)</td>
<td>22533 ± 8873</td>
<td>1771 ± 450</td>
</tr>
<tr>
<td>P-value</td>
<td>.0001</td>
<td></td>
</tr>
</tbody>
</table>
Figure 13. To quantify Pnmt expression, Pnmt<sup>+/Cre</sup>, ROSA<sub>26</sub>βgal/βgal adult mouse heart images were partitioned into right and left heart. The line of separation originates from the apex and extends toward the base of the heart and through the aorta (bold line). Additional partitioning isolates the IVS from the RVFW and LVFW (panel b and d). Each partition was then analyzed to determine the total pixels containing the blue XGAL+ stain. The left heart shows heavy XGAL+ staining when compared to the right side of the heart. Pnmt<sup>+/+</sup>, ROSA<sub>26</sub>βgal/βgal control heart shows no XGAL+ staining (panel c and d).
3D computer-aided reconstruction of the PNMT expressing cells in the mouse heart

To gain a more complete view of adrenergic cell contributions in the adult mouse heart, we examined the three-dimensional distribution of XGAL+ cells in this model utilizing customized computer algorithms to generate three-dimensional representations of the XGAL staining patterns in the adult mouse heart. Representative static views of this analysis are shown in Figure 14. The XGAL staining in the adult mouse heart appears to be largely restricted to the left side of the heart, with greater concentration near the base and mid-regions of the LV compared to the apical region. These three-dimensional views confirm the two-dimensional analyses already shown, and provide a more thorough picture of the XGAL staining distribution throughout the adult mouse heart.
Figure 14. Three-dimensional reconstruction of the XGAL+ staining in the Pnm\textsuperscript{+/-}\textsuperscript{Cre}, ROSA26\textsuperscript{+/βgal} heart. (a) Representative two-dimensional section of the adult mouse heart were cut at 20 microns. (b) Still-shot of a 3D-rendered image generated from stacked 2D images as described in the methods section. The arrows point to equivalent regions in both panels that roughly correspond to basal, mid, and apical concentrations of XGAL+ cells in these hearts. Please see online supplemental figures 1 and 2 for different perspective views of the 3D image analysis for these data.

Histochemical and immunofluorescent analyses of adrenergic-derived cells in the adult mouse heart

To obtain a more detailed view of the XGAL+ staining in the adult mouse heart, we captured microphotographs of increased magnification. Representative examples of these results show that most of the cells in the LA were stained with XGAL, especially
along the peripheral borders. Similarly, the free wall of the LV was also stained strongly for XGAL (Figure 15, panels d-f).

Figure 15. Pnmt-Cre expression in cardiomyocytes of the adult mouse heart. XGAL+ staining in the left heart reveals the anatomical location of cells which have historically expressed the Pnmt gene. Pnmt expression can be seen in the LA and the apical border of the LV (panel a). Pnmt expression is seen distributed throughout the LA and can be seen within the cardiomyocytes under higher magnification (b, c). Pnmt expression is also observed in the free wall of the LV and appears to be within the cardiomyocytes, based on anatomical and morphological characteristics (d-f).

Anatomical and morphological evaluations of the sections are consistent with a myocyte-specific pattern of staining, though it is also clear that many myocytes remain
unstained. Examples of specific myocardial staining were verified with higher magnification phase-contrast and light microscopic images (Figure 16 a and 16 c). Inverted image analysis of the stained cells where characteristic sarcomeric striations are clearly evident in the XGAL-stained cells (Figure 16 b and 16 d, arrows). In addition, we combined XGAL staining with immunofluorescent labeling of myocardial cells using an anti-sarcomeric α-actinin antibody (Figure 17). These results clearly show overlapping staining for XGAL and the myocyte marker protein, sarcomeric α-actinin (arrows), thereby confirming that Pnmt-driven XGAL+ staining is found in adult cardiac myocytes in the mouse heart.
Figure 16. High-magnification phase-contrast and light microscopy images of XGAL-stained cells in the LV free wall from an adult mouse heart. (a) Black and white phase-contrast image of XGAL-stained myocytes (arrows). (b) Color light microscopy image of an XGAL-stained myocyte (arrow). Inverse images of (a) and (b) are shown in panels (c) and (d), respectively. Note the characteristic striated rectangular-like shape of the stained cells.

In this model, XGAL+ staining demarcates active as well as historical expression of Pnmt [12]. To identify cells actively expressing Pnmt in the adult mouse heart, we used an anti-Pnmt antibody to perform immunofluorescent labeling of cardiac tissue.
sections from adult Pnmt$^{+/\text{Cre}}$, ROSA26$^{+/\beta\text{gal}}$ mice. We first tested the specificity of this antibody using adult adrenal gland sections from the same mice.

![Image of Immunofluorescent staining for sarcomeric α-actinin in the adult mouse LV (a, arrows) and an adjacent serial section (10 μm) stained with XGAL (b, arrows) indicating a history of Pnmt gene expression. Overlay of the Immunofluorescent and XGAL staining show a clear co-localization of cells with a cardiomyocyte phenotype (c, arrows).]

Figure 17. Co-localization of XGAL and sarcomeric α-actinin in LV cells of the adult mouse heart. Immunofluorescent staining for sarcomeric α-actinin in the adult mouse LV shows characteristic striations associated with cardiomyocytes (a, arrows). An adjacent serial section (10 μm) stained with XGAL shows dark (positive) staining, indicating a history of Pnmt gene expression (b, arrows). Overlay of the Immunofluorescent and XGAL staining show a clear co-localization of cells that have a history of Pnmt expression and cells with a cardiomyocyte phenotype (c, arrows).
As shown in figure 18, cells in the adrenal medulla were brightly labeled with the anti-Pnmt antibody, while the surrounding cortex displayed only background fluorescence (panel a). At higher magnification, the Pnmt immunofluorescent labeling showed characteristic cytoplasmic localization (Figure 18, panel c), as expected based on earlier immunofluorescent labeling of adrenal chromaffin cells with different anti-Pnmt antibodies used in previous studies [65, 86]. When applied to cardiac tissue, Pnmt immunofluorescent staining identified small triangular-shaped cells that primarily appeared to be located in interstitial spaces (Figure 18 b, arrows). The labeling also appeared to be largely cytoplasmic, and was likely not due to autofluorescence since little or no fluorescent signal was apparent from them upon switching to a red filter (Figure 18 d, arrows). As an additional control, no specific fluorescent staining of either adrenal or cardiac tissue sections was observed in the green spectrum in the absence of the anti-Pnmt primary antibody (not shown). These data indicate that the observed Pnmt immunofluorescent labeling is specific for cells expressing Pnmt.
Figure 18. Active Pnmt expression in the adult mouse adrenal glands and heart. The adrenal gland and heart of adult Pnmt+/Cre, ROSA26+/βgal mice were extracted to evaluate the distribution of active Pnmt expression. Cryosections of the mouse adrenal gland were immunofluorescently stained to identify Pnmt expression (green). The adrenal medulla (Med) showed bright positive staining as expected, while the cortex (Ctx) of the adrenal gland showed only background fluorescence (a). High magnification images of Pnmt immunofluorescent staining in the LV reveals small cells positive for cytoplasmic Pnmt expression in the heart (b, arrows) and adrenal medulla (c). Negative control for the LV image shown in panel b illustrates the absence of fluorescent signal in the red spectrum (d).
To explore this further, we evaluated Pnmt immunofluorescent staining in different regions of the heart as exemplified in figure 19. Positive Pnmt immunofluorescent labeling was found within the muscle layers of all four chambers of the heart, where most of them displayed the small triangular-shaped appearance (Figure 19, arrows). Some of these appeared to have process extensions, possibly suggesting neuronal-like characteristics. In fact, many process-like structures were observed in almost every section (indicated by asterisks, Figure 19).
Figure 19. Pnmt is expressed in all chambers of the adult mouse heart. Immunofluorescent staining in the adult mouse heart using an anti-Pmnt antibody shows active expression in both right and left atria (a-c). The majority of fluorescently labeled cells (green) in the atria have a triangular-shaped appearance (arrows) with neuronal-like extensions (asterisks). In addition, cardiomyocyte-like cells also show positive Pmnt staining (arrowheads) which appears to be cytoplasmic while a few cells show staining along their lateral border (d-f). The ventricular myocardium also displayed small triangular-shaped cells which were positive for Pmnt expression (e, arrows).
In a few cases, positive Pnmt immunofluorescent staining appeared to label larger brick-shaped cells in a striated pattern consistent with ventricular myocyte morphological characteristics (Figure 19 e and 19 f, arrowheads). We next performed co-immunofluorescent staining of adult mouse heart sections with anti-Pnmt and anti-sarcomeric α-actinin antibodies. When examined at high magnification using confocal laser-scanning fluorescent microscopy, it was clear that the small triangular-shaped Pnmt-positive (green) cells did not co-label with the larger brick-shaped striated myocytes in most cases, but rather were found in close association with the myocytes, typically in the interstitial spaces as shown in figure 20 (panels a and b, arrows). A few myocytes, however, appeared to also be positively stained for Pnmt, as indicated by the overlapping Pnmt and sarcomeric α-actinin staining observed (Figure 20 c, arrowheads). These results suggest that Pnmt is actively expressed in cells throughout the heart, and that a small number of these appear to be myocytes, though the vast majority of them appear to be non-myocytes.
Figure 20. High-magnification confocal co-immunofluorescent staining for Pnmt and sarcomeric α-actinin in adult mouse heart (LV) sections. Pnmt immunofluorescent staining in the adult mouse heart identifies small triangular-shaped cells (green) (a, b arrows) within the interstitial spaces. Cardiomyocyte-specific staining for sarcomeric α-actinin (red) shows identifiable striations in branching cells. Immunofluorescent co-staining with Pmnt and sarcomeric α-actinin shows apparent Pmnt expression within some cardiomyocytes (c, arrowheads).
Discussion

In the present study, we showed that cells with a history of expressing the adrenergic biosynthetic enzyme gene, Pnmt, were present in the adult mouse heart. This was confirmed by XGAL+ staining in Pnmt-Cre x R26R mice, where staining was predominantly found in the left atrial and ventricular muscle chambers. While the pattern of staining in the left atrium was rather diffuse, the distribution of XGAL+ cells in the left ventricle was clearly localized to specific regions. Notably, XGAL staining was heaviest in the basal and mid-sections of the left ventricle, and there was a defined “patch” of strongly stained cells consistently found near midline and apical sections. Staining was not exclusive to these regions, but they represent the major densities of adrenergic-derived cells in the adult heart.

It is important to note that the XGAL-stained cells reflect both active and historical expression of the Pnmt gene in the heart because Pnmt-Cre acts on the R26R locus to activate βGAL expression permanently through genetic recombination [4]. Consequently, XGAL+ cells represent the total conglomeration of cells that actively express Pnmt at the time of staining and those that had expressed it at some point earlier in development. Most of the XGAL+ cells in the adult mouse heart appeared to have myocyte-like characteristics based on their distribution, size, brick/rectangular shape, striated patterns, and overlapping staining with a well-established myocyte marker protein, sarcomeric α-actinin.
In contrast, when we exclusively examined active Pnmt-expressing cells using immunofluorescent histochemical staining methods with a specific anti-Pnmt antibody, only a relatively small number of the total Pnmt+ cells in the heart appeared to have myocyte-like characteristics. The vast majority of cardiac cells that were identified by Pnmt immunofluorescent staining were small, triangular-shaped cells located in close association with, but clearly distinct from, myocytes. These Pnmt+ cells were found in all four cardiac chambers where they were most often found in interstitial spaces between bands of myocardial cells. Several examples of process-like staining were observed in Pnmt+ cardiac cells suggesting that some of these may be neurons, though further experiments with additional cell type-specific markers are required to determine if this is true. It is nevertheless clear, however, that most of the cardiac cells identified as positive for Pnmt immunofluorescent staining do not appear to be myocytes. These results thus suggest that much of the XGAL+ staining likely represents historical expression of the Pnmt gene in addition to cells that are actively expressing Pnmt.

Previous studies have shown that extracts from the LA display relatively high levels of Pnmt mRNA and enzyme activity compared to the LV [78, 79, 87], and it has been known for many years that Pnmt and adrenaline concentrations are generally higher in the atria than in the ventricles [76, 83, 87]. This appears to be true in human patients as well [80]. Interestingly, there is also evidence indicating that Pnmt gene expression is differentially regulated in the atria compared to the ventricles [75-78]. Early studies that examined the anatomical localization of putative adrenergic cells (chromaffin-like or small-intensely-fluorescent “SIF” cells), and found them mainly in the
epicardium in the glomus or grape-like clusters of extraneuronal cells associated with cardiac ganglia [67-73]. Subsequent studies have shown that there is also a significant fraction of Pnmt-expressing cells that are non-ganglionic in both atria [78], and it is well established that chemical destruction of sympathetic nerves with 6-OHDA leads to increases in Pnmt expression and adrenaline production in the heart [83, 84]. Taken together, these previous studies have shown that non-neuronal Pnmt is expressed in the adult hearts from a number of mammalian species, including humans. Most of this expression was reported in the atria, especially the LA [78, 79]. Some of this expression may be derived from the small clusters of chromaffin-like cells found in and around cardiac ganglia, but the evidence indicates that there must also be a significant source of these cells that not associated with ganglia in the heart [77, 80]. The data presented here in combination with previous reports on the origin of adrenergic cells within the developing heart suggest that a significant fraction of these cells are derived from myocardial progenitors [12, 66, 74, 78], while others are likely derived from invading neural crest subpopulations [88, 89].

The present study is the first to demonstrate the cellular distribution of adrenergic cells in adult myocardium. Previous histological examinations focused on chromaffin or SIF cells located in and around ganglia on the epicardial surface of the heart [67-70], but no specific cellular staining for Pnmt was reported. Subsequent studies have examined Pnmt mRNA, protein, enzyme activity, and adrenaline formation in the heart [76-78, 80, 83, 84], but all of these studies relied on preparation of extracts from the heart. Thus, to the best or our knowledge, this is the first report of detailed anatomical
and histological identification of cardiac cells that express Pnmt and those that had a history of expressing Pnmt at some point in their development.

Our previous work demonstrated that adrenergic cells were transiently and progressively associated with regions of the developing heart associated with pacemaking and conduction system centers [65]. In the adult mouse heart, we observed a limited pattern of XGAL+ staining in the right atrium, atrio-ventricular junction, and along the crest and lateral borders of the interventricular septum that are generally consistent with the expected distribution of pacemaking and conduction system tissue, but the predominant pattern of XGAL+ staining in the adult mouse heart was not found in these regions. In further contrast to earlier stages of development (i.e., fetal and neonatal) where XGAL+ staining was observed extensively in the muscle cells of all four cardiac chambers [12], the adult staining pattern of Pnmt-driven XGAL staining was clearly much more restricted to myocardium on the left side of the heart.

Interestingly, the left-sided patterning of adrenergic-derived cells in the adult myocardium does not coincide with any known gene expression localization profiles; however, it appears to share anatomical similarity with the ascending segment of the helical ventricular myocardial band (HVMB) described by Torrent-Guasp et al. [35, 90]. Although controversial and not universally accepted [91, 92], the HVMB model describes the directional orientation and intricate looping of myocardium which occurs in order to form two independent ventricles that function as a single unit. The detailed looping described in the HVMB model results in a pair of apical left ventricular loops; ascending and descending, that criss-cross to form a funnel-shaped structure near the
tip of the apex. The pattern of XGAL+ staining in the apical region of the adult mouse heart suggests that adrenergic-derived cells could be a component of the ascending fibers of the HVMB model. Additional experiments are required to test the validity of this model.

The presence of adrenergic-derived cells within the adult mouse heart could also suggest involvement of these cells in stress-mediated cardiomyopathies. In particular, the cardiomyopathy known as Tako-Tsubo or “Broken-Heart Syndrome” involves hypokinesis or akinesis of the mid and apical regions of the left ventricle during periods of severe emotional stress [93-96]. Patients with Tako-Tsubo typically have symptoms that are similar to myocardial infarction (e.g., chest pain, elevated S-T segment on ECG), but do not display coronary occlusion. Due to the hypokinesis of the mid and apical regions of the left ventricular muscle these regions “balloon” out when viewed by echocardiography, lending Tako-Tsubo another alias, “Apical Ballooning Syndrome” [97, 98]. The underlying causes of this syndrome are not well understood, however, the main hypotheses that have been put forward all involve adrenergic mechanisms [99-102]. Our current findings show that adrenergic-derived cells preferentially populate specific portions of the LV free wall, thereby suggesting that these cells could play an important role in the development of certain stress-induced cardiomyopathies such as Tako-Tsubo Syndrome. It is important to note, however, that possible connections to clinical syndromes remain speculative at this point, and that additional experiments are needed to determine the validity of these ideas.
In conclusion, this work demonstrates a distinctive and unexpected left-sided distribution of adrenergic-derived myocardial cells in the adult mouse heart. The specific distribution of these cells in the heart was visualized using 2D and 3D analysis methods to gain a comprehensive map of their anatomical positions. The vast majority of these cells were found on the left side of the heart. While apparently random and uniform in the left atrium, the pattern of these cells in the free wall of the left ventricular musculature appeared in concentrated bands near the base, mid-section, and apex. This anatomical distribution could implicate adrenergic-derived left ventricular myocardium in some forms of stress-induced cardiomyopathies. Further study is required to determine the potential physiological significance of these unique findings.
REFERENCES


