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14. ABSTRACT Heart fibrosis and loss of blood vessels are prominent pathologic abnormalities in diabetics that lead to the development of heart failure. Moreover, reduced angiogenesis after a heart attack is responsible for defective myocardial repair in diabetic subjects. Although the negative impact of diabetes on the heart is widely appreciated, the cellular alterations and molecular signals involved in fibrosis and blood vessel loss in diabetes remain unknown. Applying genetic fate mapping tools, we have uncovered an unexpected plasticity and heterogeneity in reparative cells and identified common cellular links between angiogenesis and fibrosis. We investigate the role of these novel biological mechanisms in the pro-fibrotic and angiostatic effects of diabetes, focusing on the contribution of pericytes and endothelial cells in the cardiac tissue repair process.					
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Title: "Cellular Plasticity in the Diabetic Myocardium"

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First Annual Report from 09/01/2016 to 08/31/2017

1. Introduction

Heart tissue fibrosis and loss of blood vessels are prominent pathologic abnormalities in diabetics and lead to the development of heart failure. Moreover, reduced angiogenesis after a heart attack is responsible for defective myocardial repair in diabetic subjects. Although the negative impact of diabetes on heart function and repair is widely appreciated, the cellular alterations and molecular signals involved in fibrosis and blood vessel loss in diabetes remain unknown. Applying genetic fate mapping tools, we have uncovered an unexpected plasticity and heterogeneity in reparative cells and identified common cellular links between angiogenesis and fibrosis. We investigate the role of these novel biological mechanisms in the pro-fibrotic and angiostatic effects of diabetes, focusing on the contribution of pericytes and endothelial cells in the cardiac tissue repair process.

2. Keywords

Diabetes, cardiomyopathy, heart failure, fibrosis, angiogenesis, vascular rarefaction, pericytes, endothelial cells, endothelial-to-mesenchymal transition, cellular plasticity, extracellular matrix, lineage tracing, cell fate mapping, gene expression, signaling pathways

3. Accomplishments

Major Scientific Goals of the Project

The project has the following three major goals:

- a) Determine the contribution of pericytes to the development of cardiac fibrosis in diabetic mice (**AECOM**);
- b) Evaluate the role of endothelial cells in the development of cardiac fibrosis in diabetic mice (**VUMC**);
- c) Identify molecular pathways promoting fibrosis and causing blood vessel loss in diabetic hearts (**AECOM & VUMC**).

Scientific Accomplishments of the Project

a) Work performed at AECOM

We have characterized the db/db mouse as a model of cardiac fibrosis and diastolic dysfunction that recapitulates characteristics of human heart failure with preserved ejection fraction (HFpEF). Moreover, we have systematically studied gender-specific responses in this model. Our experiments demonstrated that obese diabetic db/db mice in a C57Bl6J background exhibit cardiac remodeling, associated with modest ventricular dilation, accompanied by marked left ventricular hypertrophy, in the absence of systolic dysfunction (Figure 1A-I). Elevated left ventricular end-diastolic pressure (LVEDP) in db/db mice suggests significant diastolic dysfunction (Figure 1J-L). Hypertrophic changes, chamber dilation and diastolic dysfunction are more prominent in female animals. Thus, the db/db mouse model recapitulates features of HFpEF observed in human patient populations and is particularly useful in understanding the pathogenesis of cardiac dysfunction associated with metabolic disease.

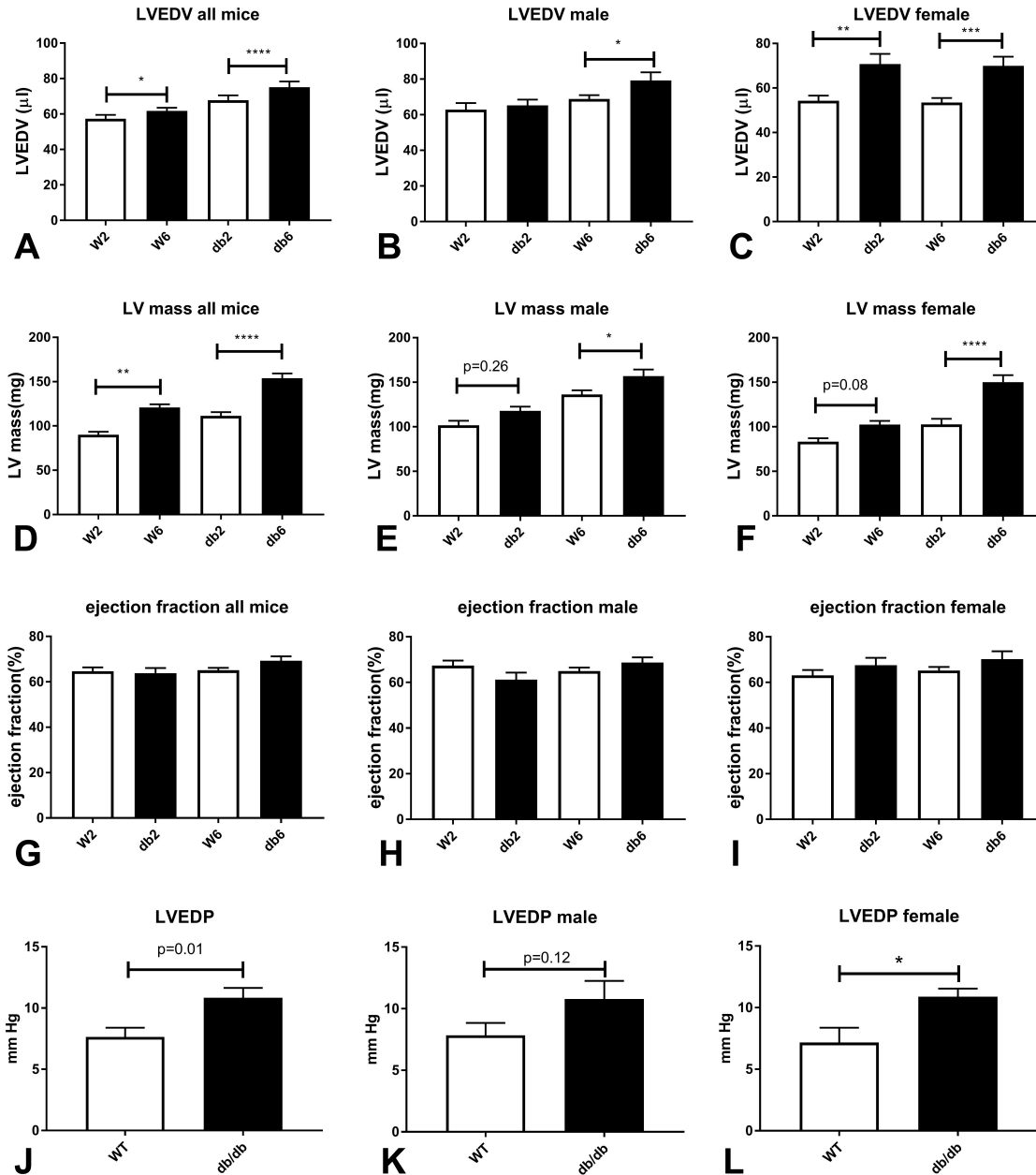


Fig. 1: The db/db mouse recapitulates features of human Heart failure with preserved ejection fraction (HFpEF). A-C: db/db mice exhibit modest dilative remodeling at 6 months of age, evidenced by an increase in left ventricular end-diastolic volume (LVEDV). D-F. LV mass is markedly increased in db/db mice. Cardiac hypertrophy is accentuated in female db/db mice. G-I: Ejection fraction is preserved in db/db mice documenting absence of systolic dysfunction. J-L: Left ventricular end-diastolic pressure (LVEDP) is increased, predominantly in female db/db mice, suggesting diastolic dysfunction (n=6-38/group).

We have documented fibroblast activation in db/db mice. In order to examine the mechanisms of fibroblast activation in diabetic mice, we have isolated fibroblasts from 4-6 month old WT and db/db hearts. db/db fibroblasts had increased baseline levels of collagen I and III transcription, but had blunted responses to TGF- β 1 stimulation (Figure 2A-B). Fibroblast activation in db/db hearts is not associated with myofibroblast conversion. In contrast to infarct fibroblasts, diabetic fibroblasts do not exhibit expression of α -smooth muscle actin (α -SMA), periostin or fibroblast activation protein (FAP). These findings suggest that diabetes stimulates an alternative activation pathway that is not associated with myofibroblast conversion.

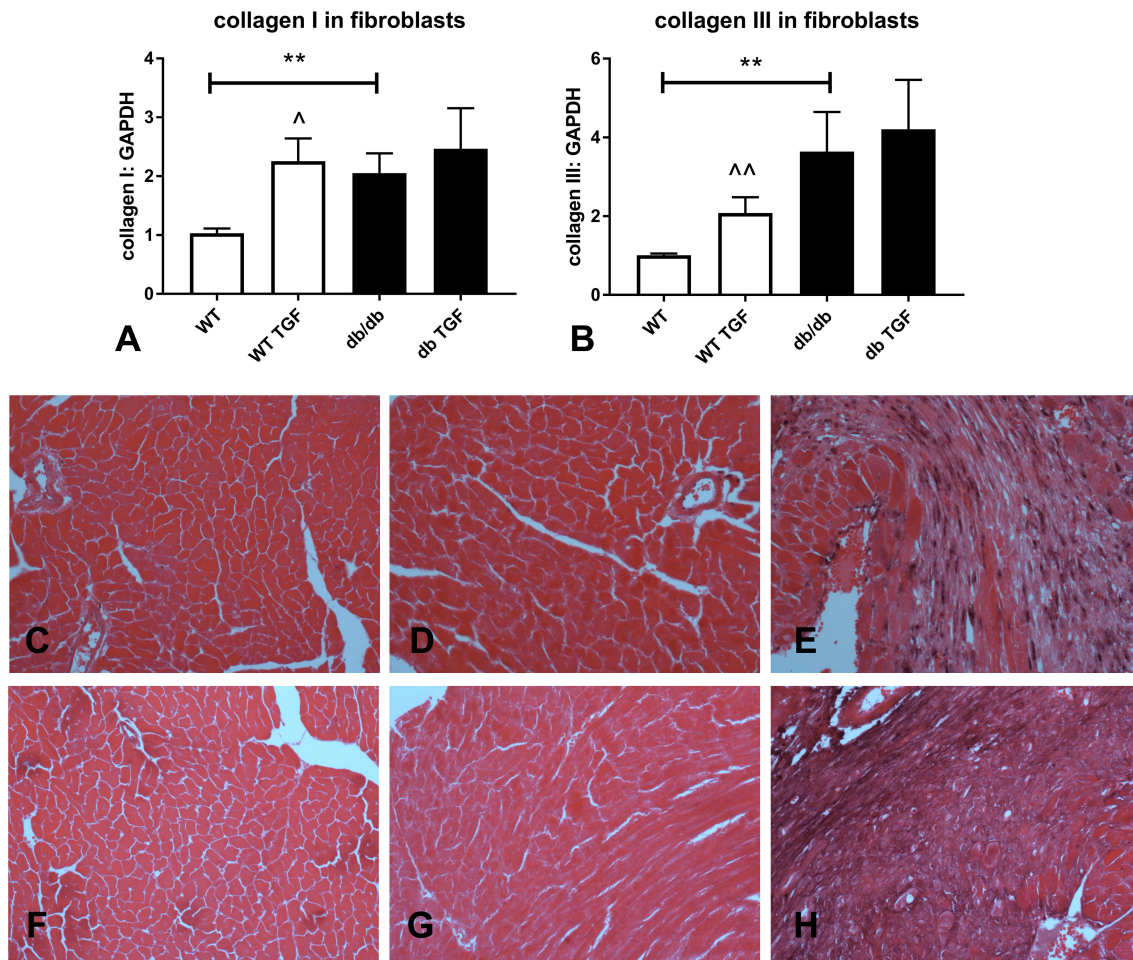


Fig 2: Activation of diabetic fibroblasts does not involve myofibroblast conversion. A-B: Cardiac fibroblasts harvested from db/db mice (at 4-6 months of age) exhibit increased baseline transcription of collagen I (A) and collagen III (B). However, the response of diabetic fibroblasts from 4-6 month old mice to TGF- β 1 is blunted (n=6/group). C-H. Activation of fibroblasts in the diabetic heart does not involve myofibroblast conversion.

Immunohistochemical staining for fibroblast activation protein (FAP, panels C-E) and periostin (F-H) in hearts from lean WT mice (C, F) and db/db mice (D, G) and in lean mouse infarcts (after 7 days of coronary occlusion). Infarcted mouse hearts exhibit infiltration with activated FAP and periostin-expressing myofibroblasts. In contrast, periostin and FAP are not expressed in diabetic hearts.

We have assessed expression of TGF- β -related genes in db/db mouse hearts.

Because TGF-betas are critically involved in the fibrotic response and are activated in a high-glucose environment, we performed a comprehensive analysis of the effects of diabetes on TGF- β superfamily genes using a PCR array. We found that at the 6 month timepoint, TGF- β isoforms is not significantly upregulated in the diabetic myocardium; however expression of the T β RII receptor and the inhibitory pseudoreceptor BAMBI is markedly higher. Moreover, BMP4, BMP6 and Bmpr2 exhibit significant upregulation (Figure 3). The findings highlight the complex regulation of TGF- β cascades in vivo. Early induction of TGF- β s may be followed by activation of endogenous inhibitory pathways (such as the decoy receptor BAMBI), in order to restrain TGF- β -driven fibrosis.

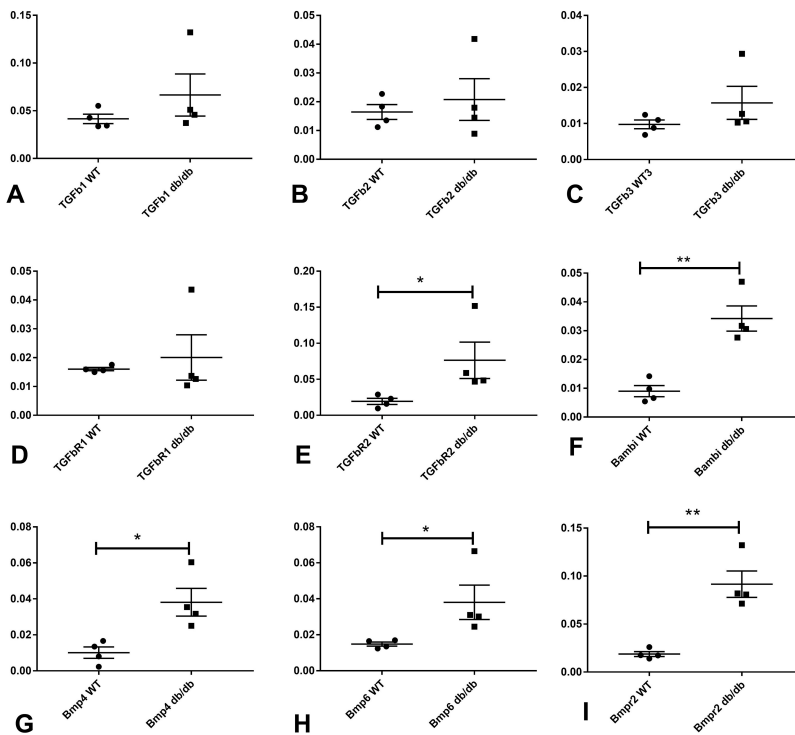


Fig 3: Induction of TGF- β superfamily genes in the diabetic heart. PCR array was used to assess gene expression in male mouse hearts at 6 months of age (n=4). TGF- β isoforms

were not significantly upregulated in db/db mice; however expression of the T β RII receptor and the inhibitory pseudoreceptor BAMBI were markedly elevated. There was also evidence for activation of the BMP cascade. BMP4, BMP6 and Bmpr2 expression levels were significantly higher in db/db hearts.

We are using lineage tracing strategies to test the hypothesis that cardiac pericytes generate fibroblasts in the diabetic myocardium. We have crossed db/+ mice with NG2-*dsred* pericyte reporter animals to generate diabetic pericyte reporter mice. db/+; NG2*dsred* animals are bred with db/+ controls, in order to generate db/db;NG2*dsred* mice (12.5% of the offspring). Unfortunately breeding of 2 NG2*dsred* animals appears to significantly decrease litter size; thus generation of adequate numbers of diabetic pericyte reporter mice will require expansion of our colonies. For lineage tracing, NG2-Cre mice were crossed with R26R*stopYFP* (Rosa-YFP) mice in order to generate double transgenic NG2-Cre-YFP mice to specifically label pericytes and their progeny. We currently have 12 breeding cages of db/+; NG2-Cre; Rosa-YFP to generate homozygote db/db mice that are also positive for NG2-Cre Rosa-YFP. We have already generated 6 diabetic mice, positive for NG2-Cre and Rosa-YFP that can be used for lineage tracing. The first experimental material from these animals will be available in 2 months.

b) Work performed at VUMC

We are using Cre/Lox-based cell lineage tracing strategies to evaluate the contribution of endothelial cells to the generation of extracellular matrix producing cells in diabetic mouse hearts, thereby contributing to interstitial fibrosis and myocardial dysfunction. To this end, we have crossed the Tie1-Cre mice to the R26R*stopYFP* (Rosa-YFP) mice in order to generate double transgenic Tie1-Cre-YFP mice to specifically label endothelial cells and their progeny. These mice were then bred to each other and genotyped in order to generate Tie1-Cre-YFP mice that are homozygote for both the Cre recombinase and R26RYFP loci. Double homozygote Tie1-Cre-YFP mice were then bred to db/+ heterozygotes to generate db/+ Tie1-Cre Rosa-YFP mice and non-diabetic Tie1-Cre Rosa-YFP siblings as controls. We have obtained approximately 25 db/+ heterozygotes and 30 non-diabetic controls that are also heterozygote for both the Tie1-Cre Rosa-YFP loci. We are currently breeding db/+ Tie1-Cre Rosa-YFP mice to generate homozygote db/db mice that are also positive for Tie1-Cre Rosa-YFP. At

present, 5 breeding pairs are successfully breeding. Five newly added cages (total of 10 breeding pairs) are expected to expand the number of experimental animals with desired genotypes to 10-15 mice, which will be sufficient to perform the endothelial cell lineage tracing experiments.

c) Work performed collaboratively at VUMC and AECOM

To identify novel molecular pathways linked to pathological fibrosis and vascular rarefaction in diabetics, we compared total heart gene expression profiles between female control and diabetic mice at 6 months of age, where there is clear functional and histological evidence of diabetic cardiomyopathy. In brief, hearts from 3 normal and 3 db/db mice were isolated at **AECOM** and shipped to **VUMC** where total RNA was extracted using standard techniques. The 6 RNA samples were then sent to the Vanderbilt sequencing core (VANTAGE). 5 of 6 samples (3 db/db and 2 normal controls) passed the strict RNA quality control and processed for RNA sequencing. A portion of the purified and validated RNA samples that passed quality controls was shipped from **VUMC** back to **AECOM** for analysis using TGF- β superfamily gene PCR arrays (please see **section a** above)

Paired end sequencing was performed at 75X depth with 45 million reads per sample. Raw data (FASTQ files) were uploaded to Partek Flow for analysis and data quality assessment. Sequences were aligned to the mm10 platform of the *Mus musculus* genome using STAR, followed by total count normalization with minimum values set to a minimum of 0.0001. Data were annotated using Ensemble Release 83 and quantified at the gene level using the Partek E/M multimodel algorithm. Partek Genomics Suite 6.6 was used for principal components analysis and for hierarchical clustering with average linkage and Euclidian distance of normalized values (RPM) generated from BAM files (i.e., aligned sequences).

As shown in Figure 4, diabetic heart samples clustered separately from controls, with 2,269 transcripts showing significant differences between the two genotypes. In

total, 310 genes showed higher and 1,959 genes showed lower expression levels in diabetic hearts compared to normal controls.

Data were submitted to Gene Set Enrichment Analysis (GSEA) at the Broad Institute and to DAVID (Database for Annotation, Visualization and Integrated Discovery). Up and down-regulated processes were analyzed using DAVID and gene ontology. GSEA provided signaling pathways derived from the RNA sequencing data and associated genes.

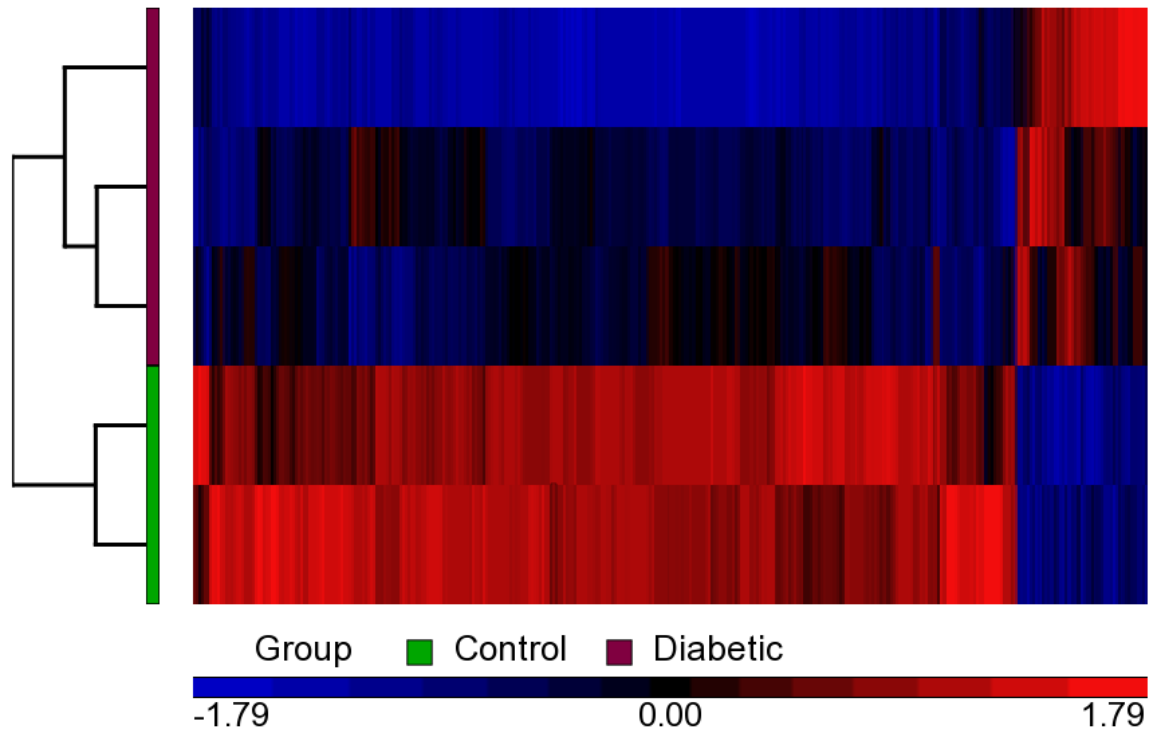


Fig 4: Hierarchical clustering of 2,269 RNA transcript reads per kilobase per million mapped reads for genes with significant differential expression (fold difference >1.5 , p value <0.05) between 2 control and 3 diabetic samples using Partek Genomics Suite with average linkage and Euclidean distance measures. Bright red, bright blue, and black indicate the highest, lowest, and median normalized reads, respectively. Vertical dendrograms represent the individual tissue samples (green = control, purple = diabetic).

The results showed that many upregulated transcripts represent genes expressed during heart development (Figure 5), a finding that is consistent with previous reports indicating activation of the fetal gene expression program in failing diabetic hearts. Moreover, there is upregulation of extracellular matrix (ECM) proteins, indicative of fibrosis. Among key signaling pathways, we found activation of Wnt signaling

components, suggesting a role of the pathway in defective angiogenesis and fibrosis in diabetic hearts. In contrast, the majority of the downregulated pathways suggest abnormal immune system response and deregulation of catabolic pathways (Figure 6).

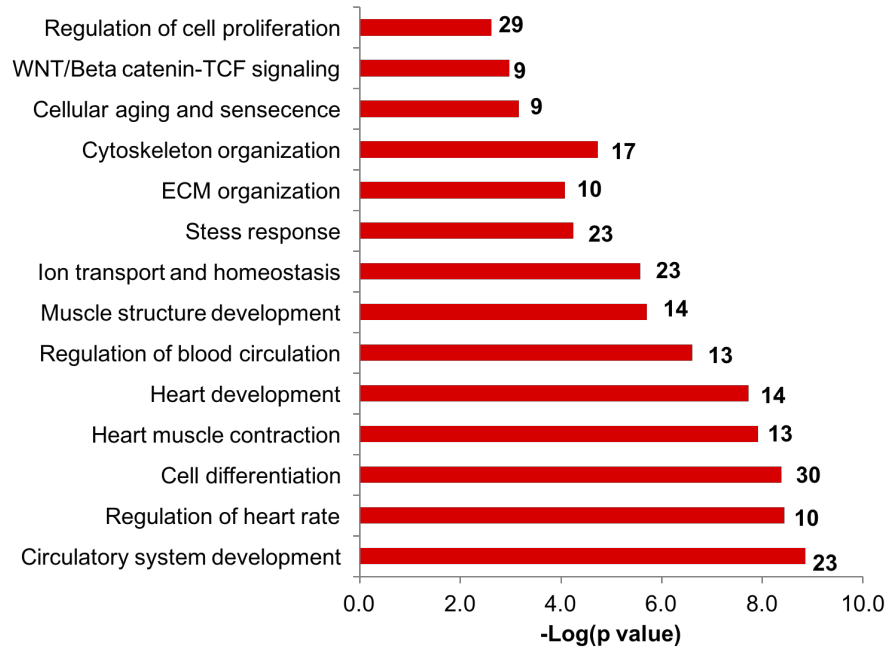


Fig 5: Chart showing key up-regulated pathways in diabetic hearts. The number of genes in each pathway is indicated to the right.

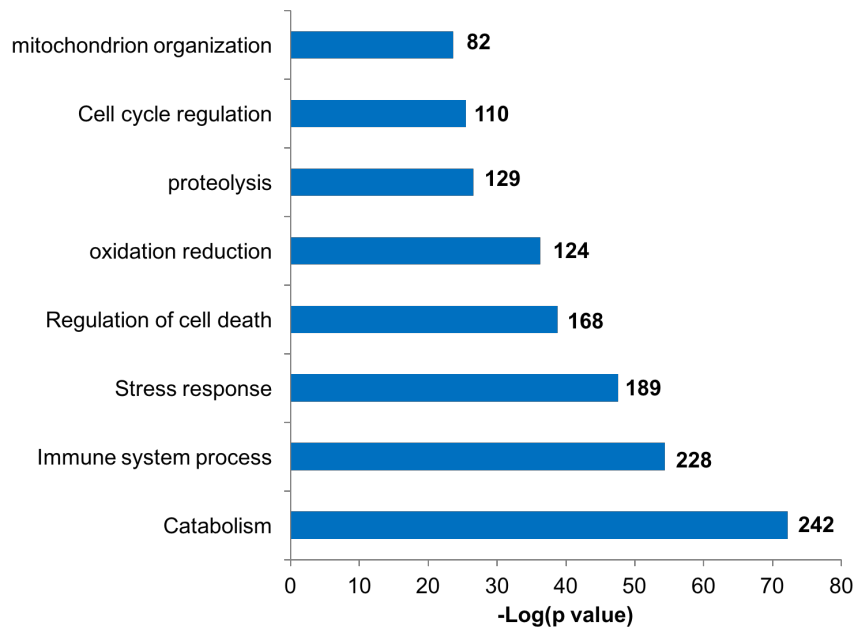


Fig 6: Chart showing key down-regulated pathways in diabetic hearts. The number of genes in each pathway is indicated to the right.

Training opportunities and professional development

Cassandra Awgulewitsch, graduate student, has joined the project. She has just completed her second year of graduate training. She successfully passed her PhD Thesis qualifying exams in August 2017 and has officially joined the Cell and Developmental Biology Training Program. Ms. Awgulewitsch has received a fellowship from the NIH-sponsored T32 training grant “Program in Cardiovascular Mechanisms: Training in Investigation” (T32HL007411). Since joining our laboratory, she has published a first author review article on the role of endothelial cell plasticity in homeostasis and disease (see “Products” section below), and co-authored a manuscript on the role of BMP signaling inhibition in cardiomyocyte differentiation (Bylund et al., *Stem Cells & Dev.*, 2017).

The primary training of Cassandra is laboratory work under the direct supervision of Dr. Hatzopoulos. Cassandra is currently directs the breeding of Tie-1-Cre-YFP mice to db/+ heterozygotes to generate diabetic and non-diabetic Tie1-Cre Rosa-YFP mice to perform lineage tracing experiments. She has also spearheaded the RNAseq data analyses (Figures 4-6). Her training includes daily informal interactions, weekly data review in face-to-face meetings, and weekly laboratory meetings and journal clubs. A second essential component of the training program involves participation in the Journal Clubs organized weekly by the Cell & Developmental Biology Department throughout the academic year and Work-in-Progress Cardiovascular Research Seminars (CVRS). The CVRS meetings involve formal seminars by graduate students and post-doctoral fellows, giving trainees an excellent opportunity to organize their findings, develop and defend hypotheses, and refine their communication skills. There are also numerous departmental seminars each week in the VUMC campus from prominent visiting investigators, often closely related to Cassandra’s scientific interests.

In the coming years, Cassandra will have the opportunity to take part in local annual scientific meetings such as the Graduate Student Research Symposium (GSRS), the Program in Developmental Biology retreat and the Cardiovascular Research Day at VUMC. These meetings bring nationally recognized scientists for seminars exposing trainees to the latest in contemporary research and giving students an opportunity to

present their own work to a broader audience as posters, or short oral presentations. She will also be encouraged to submit abstracts to large national meetings such as the American Heart Association Scientific Sessions, Keystone Symposia, etc. Finally, Cassandra has the opportunity to supervise the work of junior investigators in the laboratory (e.g., rotating graduate students, undergraduates and high school students), which will help with development of leadership and mentoring skills.

How were the results disseminated to communities of interest?

Most of the findings presented in this report are preliminary; these were not yet disseminated to the general scientific community and public, except during informal presentations and discussions in internal work-in-progress meetings and regular teleconferences between the partnering institutions.

Plans for the next reporting period

During the next reporting period, we expect to perform a significant part of the planned cell lineage tracing experiments. The time consuming breeding period to achieve the desired triple genotypes is almost complete. Therefore, we expect to analyze hearts from 2 and 6 months old mice using primarily histological techniques to assess the extent of endothelial-to-mesenchymal transition contribution to vascular rarefaction and fibrosis. Furthermore, the contribution of pericytes in fibrosis and the pathophysiology of diabetic cardiomyopathy will be assessed using the corresponding Cre lines.

We will validate the primary RNAseq data using histological, molecular and cellular techniques. These analyses will likely yield new insights in the development of diabetic cardiomyopathy and may identify new targets to treat heart disease in diabetics. We are particularly excited about the putative role of Wnt signaling, because our previous work has shown that Wnt signaling is activated in endothelial cells and fibroblasts during neovascularization and scar formation after acute ischemic injury (Aisagbonhi et al., Disease Models Mech. 2011; Paik et al., Circ. Res. 2015).

Our preliminary data also show significant differences in gene activity in diabetic hearts between female and male mice. Because women suffer more severe diabetic cardiomyopathy than men, we plan to systematically compare gender-specific gene expression profiles, expanding the RNAseq analysis to male hearts at comparable ages to females. We will also perform independent lineage tracing studies in male and female mice. We expect these original studies to provide novel information about gender-specific deficits in diabetes.

4. Impact

HFpEF is a major cause of morbidity and mortality worldwide. There is currently no effective treatment for patients with HFpEF. Although cardiac fibrosis has been implicated in the pathogenesis of HFpEF, the cellular basis for fibrotic remodeling of the ventricle is poorly understood. Metabolic diseases (such as obesity and diabetes) are associated with an increased incidence of HFpEF; however, the pathophysiological mechanisms responsible for this association remain unknown. Our experiments have established a model of HFpEF due to metabolic disease that can be used to dissect cellular mechanisms. This is of outstanding significance for pathophysiologic dissection in vivo. Our planned experiments will use lineage tracing strategies, in vivo and in vitro approaches to dissect the basis for activation of diabetic fibroblasts. The significance of the studies extends beyond the cardiovascular field, as diabetes-associated tissue fibrosis has an impact on other organs (such as the kidney and the liver).

5. Changes/Problems

No changes or problems to report

6. Products

Publications

CP Awgulewitsch, LT Trinh and AK Hatzopoulos. The vascular wall: a plastic hub of activity in cardiovascular homeostasis and disease. *Current Cardiology Rep.* 2017, 19:

51. ***Acknowledgment of federal support: Yes***

7. Participants & Other Collaborating Organizations (VUMC)

Participants

Name:	<i>Antonis Hatzopoulos</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>0060562</i>
Nearest person month worked:	<i>3</i>
Contribution to Project:	<i>Dr. Hatzopoulos designed experiments, supervised the experimental work, and took part in data analyses.</i>
Funding Support:	

Name:	<i>Sara Block</i>
Project Role:	<i>RA II</i>
Researcher Identifier (e.g. ORCID ID):	<i>0133902</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Ms. Block initiated the breeding and genotyping of the diabetic db/db mice. She left in October 2016 to become Core Manager of the Thoracic Biorepository Laboratory in the Vanderbilt-Ingram Cancer Center.</i>
Funding Support:	

Name:	<i>Awgulewitsch, Cassandra</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	<i>0130163</i>
Nearest person month worked:	<i>9</i>
Contribution to Project:	<i>Ms. Awgulewitsch has participated in the generation of diabetic Tie1Cre-YFP mice to lineage trace endothelial cells. She also took part in the generation and Bioinformatics analysis of RNAseq data from diabetic and control hearts. She currently analyzes the effects of high glucose on endothelial cells and induction of endothelial-to-mesenchymal transition.</i>
Funding Support:	<i>Ms. Awgulewitsch is supported by a T32 Training Program in "Cardiovascular Mechanisms: Training in Investigation" (HL007411) fellowship.</i>

Name:	<i>Trinh, Linh ThiThuy</i>
Project Role:	<i>RA I</i>
Researcher Identifier (e.g. ORCID ID):	<i>0138489</i>
Nearest person month worked:	<i>5</i>
Contribution to Project:	<i>Ms. Trinh took over from Ms. Block and participated in the breeding and genotyping of the Tie1-Cre-YFP mice . She also isolated and performed quality controls on cardiac RNA samples from control and db/db mice that were then analyzed by RNAseq in the VUMC Vantage Core. She has also tested and validated a panel of antibodies to monitor fibrosis and vascular density in diabetic mouse hearts using immunohistochemistry on paraffin sections. Ms. Trinh left in May 2017 to attend graduate school.</i>
Funding Support:	

Name:	<i>Cannon-Stewart, Presley</i>
Project Role:	<i>RA III</i>
Researcher Identifier (e.g. ORCID ID):	<i>0146903</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Ms. Cannon-Stewart is testing a panel of antibodies to monitor fibrosis and vascular density in diabetic mouse hearts using immunofluorescence on cryosections. She is also establishing cell isolation protocols from cardiac tissue to quantify endothelial and collagen-producing cells. She continues the work of Ms. Linh.</i>
Funding Support:	

Name:	<i>Park, Matthew</i>
Project Role:	<i>Undergraduate Student</i>
Researcher Identifier (e.g. ORCID ID):	<i>0137141</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Mr. Park assisted in Bioinformatics analyses.</i>
Funding Support:	

Name:	<i>Zhu, Amy</i>
Project Role:	<i>Undergraduate Student</i>
Researcher Identifier (e.g. ORCID ID):	<i>0137371</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Ms. Zhu has participated in experiments to study the effect of diabetes and wnt signaling on inflammatory cells.</i>
Funding Support:	

Active other support of the PI (Antonis Hatzopoulos)

ACTIVE

5R01HL122417 (PI: Hemnes) 01/01/2015-12/31/2019 1.20 calendar months
NIH/NHLBI \$333,310
Lipid Deposition in the Right Ventricle in Pulmonary Arterial Hypertension

R01GM114640 (PI: Thompson) 09/01/2016-04/30/2017 1.20 calendar months
NIH/NIGMS \$51,675
Structure-Function Investigation of DAN-mediated BMP Antagonism

U01HL099997 (PI: Hatzopoulos) 09/01/2016-04/30/2018 0.60 calendar months
NIH/NHLBI \$31,847
Functional heterogeneity in cardiac progenitor cells

1R01HL138519 (PI: Hatzopoulos) 07/01/2017-06/30/2021 3.00 calendar months
NIH/NHLBI \$250,000
Functional heterogeneity of cardiac reparative cells after injury

COMPLETED

U01 HL100398 (PI: Hatzopoulos) 9/30/2009-04/30/2017 5.40 calendar months
NIH/NHLBI \$107,141
Optimizing Cardiovascular Stem Cells for Cardiac Repair and Regeneration

PENDING

None

OVERLAP

None

Collaborating Organization

Albert Einstein College of Medicine (AECOM)

1300 Morris Park Ave.

Bronx, NY 10461

Collaborating PI: Dr. Nikolaos Frangogiannis

Collaboration as described above

8. Special Reporting Requirements

N/A

9. Appendices

Awugulewitsch et al., Current Cardiology Reports article

The Vascular Wall: a Plastic Hub of Activity in Cardiovascular Homeostasis and Disease

Cassandra P. Awgulewitsch^{1,2} · Linh T. Trinh^{1,2} · Antonis K. Hatzopoulos^{1,2}

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Abstract

Purpose of Review This review aims to summarize recent findings regarding the plasticity and fate switching among somatic and progenitor cells residing in the vascular wall of blood vessels in health and disease.

Recent Findings Cell lineage tracing methods have identified multiple origins of stem cells, macrophages, and matrix-producing cells that become mobilized after acute or chronic injury of cardiovascular tissues. These studies also revealed that in the disease environment, resident somatic cells become plastic, thereby changing their stereotypical identities to adopt proinflammatory and profibrotic phenotypes.

Summary Currently, the functional significance of this heterogeneity among reparative cells is unknown. Furthermore, mechanisms that control cellular plasticity and fate decisions in the disease environment are poorly understood. Cardiovascular diseases are responsible for the majority of deaths worldwide. From a therapeutic perspective, these novel discoveries may identify new targets to improve the repair and regeneration of the cardiovascular system.

Keywords Endothelial-to-mesenchymal transition · Cellular plasticity · Vascular wall · Perivascular cells · Stem cells · Cardiovascular homeostasis

This article is part of the Topical Collection on *Regenerative Medicine*

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Introduction

In the last decade, a number of discoveries have revolutionized our view about how the cardiovascular system maintains its structure during homeostasis, repairs itself after acute injury, or responds to chronic pathological conditions such as atherosclerosis, hypertension, or heart failure. Specifically, the application of cell lineage tracing techniques—which were originally designed to identify the contribution of various cell types in organ development—has uncovered multiple origins of seemingly similar cell types, including stem cells, macrophages, and extracellular matrix (ECM)-producing cells that become mobilized to repair cardiovascular tissues [1–5]. They have documented that resident mature cells change their stereotypical identities to adopt proinflammatory and profibrotic phenotypes in the disease environment and also assume stem cell characteristics, thereby contributing to tissue maintenance under normal conditions [3, 6]. These findings have painted a new complex landscape of cardiovascular biology in adults. In this landscape, the vascular wall has emerged as the central hub of activity with endothelial cells (ECs) in the vascular wall intima, smooth muscle cells (SMCs) in the media, and perivascular progenitor cells in the adventitia showing a wide gamut of plasticity and differentiation potential. These recently recognized processes present new challenges to understand organ maintenance, but they also offer new opportunities to moderate disease progression, improve cardiovascular tissue repair, and promote regeneration.

Lineage Tracing or Cell Fate Mapping

Lineage tracing, also called cell fate mapping, is based on genetically tagging the genome of cells in permanent fashion with a gene expressing a fluorescent or other color marker. In this way, tagged cells and their progeny, no matter which cell types they

become, or how they respond to environmental conditions, can be followed throughout the organism [7, 8]. Genetic tagging depends on two components that are typically generated as two independent transgenic mouse lines. The first line is designed to express a recombinase enzyme, usually Cre in mice, under the regulatory elements of an endogenous gene promoter [9]. This is accomplished by embedding the Cre recombinase coding sequences within a gene locus or by creating a transgenic construct with Cre expression under the regulatory control of specific transcriptional elements. Depending on the gene locus and promoter/enhancer elements chosen in the design, Cre recombinase is expressed in select cell types, for example endothelial cells or cardiomyocytes [10, 11].

The second mouse line takes advantage of the ubiquitous ROSA locus that was originally described by the group of Philippe Soriano [12]. The ROSA locus is engineered to express a fluorescent or color marker such as green fluorescence protein or β -galactosidase in all cell types, but expression is blocked by a STOP signal [13]. The STOP signal is flanked by LoxP sites that can be recombined by Cre recombinase to remove the STOP signal, thus allowing expression of the marker gene in the cell where recombination took place. Expression is maintained in all of this cell's progeny, because the transcriptional elements of the ROSA locus are active in most cell types.

This technique allows precise tagging of cells based on their characteristic gene expression profiles and has provided an improved tool to trace cell lineages compared to previously used methods that were based on localized infection of cells with genetically modified viruses expressing traceable gene markers, or simple injection of carbon particles and other dyes to locally mark and follow cells during development.

Many variations on this basic theme have been developed over the years, including inducible recombinases like CreER^T that are activated by tamoxifen, thus allowing cell labeling at a defined time point [14, 15]. Inducible tagging is especially useful to lineage trace cells in adult mice, avoiding cell labeling during embryonic development, or for pulse chase experiments at distinct time points during disease progression. Another powerful tool is the Confetti mouse, which carries a multicolor reporter with four distinct fluorescent protein genes (red, yellow, nuclear green, and membrane-bound cyan) in the ROSA locus [16]. The fluorescent protein coding sequences are organized in tandem among alternating LoxP sites in a way that recombination of the ROSA-Confetti allele leads to stochastic expression of any of the four distinct fluorescent proteins. The construct is designed such that random recombination activates only one of the fluorescent protein genes, randomly labeling each targeted cell and its descendants with the same single color. As a result, this fate mapping strategy can distinguish cells that are clonally related, i.e., generated from a single originating cell versus independently derived. This powerful technique can also minimize the possibility that

cells were falsely labeled by ectopic Cre expression or Cre transfer between cells due to fusion. Alternative multi-color lineage tracing methods using the Rainbow mice have also been developed for clonal analyses [17, 18].

However, it should be noted that these techniques have several limitations that might lead to false conclusions. Most of these artifacts can be attributed to the promoter used to drive recombinase expression. Ectopic promoter expression or leaky or weak promoters can label the wrong cell types, or just a random subpopulation of relevant cells, thus leading to over- or underestimation of the contribution of different cell types to a biological process. Moreover, embryonic labeling, fusion, or genetic material transfer from cell to cell might lead to artificial labeling and incorrect inferences about genuine cellular progeny. Safeguarding against such potential artifacts by using multiple, independent, cell-specific and strong promoters, inducible Cre recombinases, Cre antibodies, pulse-labeling and chasing experiments, multicolor labeling for clonal analyses, and comparing lineage tracing results to mice expressing gene markers under the same promoter without Cre recombination is required to address these concerns and validate experimental conclusions [19•].

Despite these limitations, application of lineage tracing tools in adult disease models has broadened our perspective about how cells behave under pathophysiological conditions and revealed an amazing plasticity, especially for cells located within and around the vascular wall of blood vessels.

Intima Layer—Endothelial Cell Plasticity

The quintessential plastic cell type in the vascular wall is the endothelial cells that line up the inner layer of all blood vessels in the body. The plasticity of endothelial cells has been first recognized in the embryo where it was discovered that hematopoietic stem cells emerge from endothelial cells in the ventral wall of the dorsal aorta and subsequently colonize a succession of hematopoiesis sites in the liver, the spleen, and finally the bone marrow [20–25]. The properties of the hemogenic endothelium have been recapitulated in vitro using human iPS cell-derived endothelial cells [26].

A second site where endothelial cells give rise to a different cell type is in the developing valves and septa in the heart [6, 27, 28]. During cardiac development, endothelial cells lining the endocardial cushions undergo endothelial-to-mesenchymal transition (EndMT), giving rise to valvular interstitial cells that proceed to migrate, colonize the cardiac jelly within valve tissue, and become the major extracellular matrix (ECM)-producing cell type in the developing and mature valves.

Endothelial-to-Mesenchymal Transition

EndMT is a specialized form of epithelial-to-mesenchymal transition or EMT [6, 29]. During this process, epithelial cells

lose expression of E-cadherin, or VE-cadherin in the case of endothelial cells, and replace them with N-cadherin. Either E-cadherin or VE-cadherin proteins are present in cellular junctions and required for cell-cell adhesion; thus, their suppression loosens cells within the epithelial or endothelial layers, allowing them to become mobile and migrate [30, 31]. EndMT is also accompanied by loss of typical endothelial-specific markers, such as CD31 and vWF [30]. The downregulation of endothelial properties is followed by upregulation of genes characteristic of mesenchymal cells such as SMA, Vimentin, Periostin, FSP-1, and PDGFR α/β as well as reorganization of the cytoskeleton that transforms endothelial cells to fibroblast-like cells [30]. The initial EndMT step of downregulation of cadherin expression is accomplished by a number of transcriptional repressors such as Snail, Twist and Slug [32–34]. These repressors are direct targets of EndMT-promoting pathways such as TGF β /BMP, Wnt, and Notch signaling [33–37, 38, 39].

EndMT has now been recognized as taking place in various diseases, especially during fibrosis after acute injury or because of chronic pathological conditions [6, 29, 40]. Numerous studies using lineage tracing of endothelial cells have revealed that a significant portion of ECM-producing cells in various pathological conditions is of endothelial origin (estimates vary but usually range between 20 and 30%). Specifically, this has been shown in various models of lung fibrosis and pulmonary hypertension [41–44]; kidney fibrosis after acute or chronic injury [45–47]; the heart after acute ischemic injury such as myocardial infarction and chronic fibrosis caused by constant pressure overload [38, 48, 49, 50]; intestinal fibrosis [51]; stiffening of the arterial wall in hypertension [52]; plaque formation in atherosclerosis [53, 54]; vascular graft stenosis [55, 56]; vascular calcification [57]; diabetic retinopathy [58]; valve thickening [59]; and the generation of fibroblast-like cells that support tumor growth [60]. However, the field is not without controversy, since other studies have refuted the extent of EndMT contribution in fibrosis and pointed to proliferation and activation of resident fibroblasts as the main source of myofibroblasts after acute or chronic injury [61, 62].

Furthermore, EndMT has been identified as the cause of inherited or sporadically occurring cerebral cavernous malformations that lead to brain hemorrhages [63]. Cerebral cavernous malformations are vascular lesions that manifest when tight endothelial cell connections, which are required to maintain the integrity of the blood-brain barrier, break down due to acquisition of mesenchymal properties by endothelial cells.

Endothelial-to-Mesenchymal Transition Yields Mesenchymal Stem Cells

A fascinating aspect of EndMT is that besides yielding ECM-producing cells, it can also yield mesenchymal cells with stem

cell characteristics (MSCs) [34, 64]. At the moment, the relationship between the generation of profibrotic and regenerative cells is not clear. It is possible that EndMT is a naturally occurring phenomenon that is required for tissue homeostasis. It is also likely that this same process under various stress conditions turns functional tissue to scar formation as a necessary step to prevent tissue rupture, for example in the heart after myocardial infarction, or becomes maladaptive during chronic pathological conditions or aging [1].

Consistent with this scenario, we have recently discovered using lineage tracing that endothelial cells give rise to smooth muscle cells in the medial layer of coronary arteries, stem cell antigen-1 (Sca-1, alternatively known as lymphocyte antigen 6 complex, locus A, or Ly-6A) progenitor cells in the adventitial layer, and ventricular cardiomyocytes during cardiac homeostasis [19]. Endothelial cell-derived cardiomyocytes were organized in clonal clusters, presumably of single-cell origin. Pulse-chase experiments showed that generation of individual clusters is rapid and efficient. These new endothelial cell-derived cardiomyocytes comprise approximately 0.3% of the total cardiomyocyte population, but they are confined to specific regions of the heart in the right and left ventricular walls and the junctions between the two ventricles [19]. Endothelial derived-Sca1⁺ progenitor cells in the adventitia of coronary arteries constitute about 30–40% of the total Sca1⁺ population. Interestingly, the perivascular Sca1⁺ cells are the only endothelial progeny with high proliferation rates. The endothelial cell-fate maps are remarkably similar to those obtained by lineage tracing of Sca1⁺ cells, suggesting that endothelial cells or endothelial-like cells are upstream of Sca1⁺ progenitors [19, 65]. In support of these findings in the adult heart, a recent study showed that endothelial cells are a source of smooth muscle cells and pericytes in the heart during development [66].

Besides cardiomyocytes, endothelial cell progeny in other tissues include osteogenic cells, adipocytes, and skeletal muscle [29, 67–69]. This plasticity of endothelial cells in various tissues may be linked to their potential to transdifferentiate to multipotent MSCs.

Endothelial-to-Mesenchymal Transition Activation

What triggers EndMT in disease? Vascular inflammation has been implicated as a key inducer of EndMT in pathological conditions, and EndMT may be an adaptive response to endothelial injury [30, 51, 53, 55, 70, 71]. Shear stress is also known to induce EndMT [72]. Vascular inflammation stimulates signaling pathways such as TGF β /SMAD and Wnt/ β -catenin, which are key inducers of EndMT [38, 48, 73–77]. At the molecular level, evidence shows that FGF suppresses TGF β signaling and EndMT [78]. FGF binding to FGFR1 leads to phosphorylation and activation of ERK signaling that in turns induces expression of miR *let-7b* [55]. *Let-7b* blocks

TGF β signaling by suppressing expression of TGF β and its receptor TGF β R1. Inflammatory cytokines induced after acute or in chronic injury inhibit FGFR signaling, reducing *let-7b* levels, further potentiating TGF β signaling in endothelial cells and, thus, inducing EndMT.

Inflammation and shear stress have been shown to activate expression of the transcription factor Kruppel-like factor 4 (KLF4), which is known to promote TGF β /BMP signaling in endothelial cells [79]. KLF4 activation has been recently identified as the major inducer of EndMT in cerebral cavernous malformations, suggesting that KLF4 might be a key trigger of pathological EndMT [80, 81]. Additional regulators of EndMT include Notch signaling, several miRs, endothelin 1, Tie1 tyrosine kinase receptors, and sonic hedgehog signaling, providing a wide range of potential targets to regulate EndMT in pathological conditions [29, 37, 82–87].

Media and Adventitia—Smooth Muscle and Perivascular Cells

Although most of our knowledge about cell plasticity in disease has so far come from studies on endothelial cells and EndMT, several reports have shown that smooth muscle cells (SMCs) in the media of blood vessels and adventitia perivascular cells have also the potential to transform to other cell types and acquire proinflammatory and profibrotic phenotypes.

Studies have hinted to the plasticity of SMCs in atherosclerotic lesions and their transformation to macrophage-like cells in both mouse models and atherosclerotic lesions in human patients [88, 89]. Recent studies using SMC-specific promoters such as *Myh11* and *SM22* to mark and lineage trace SMCs within atherosclerotic lesions found that SMCs lose their characteristics and give rise to cells that exhibit phenotypes of other cell lineages, including macrophages and mesenchymal stem cells (MSCs) [90, 91•, 92]. Interestingly, using Confetti mice, it was shown that SMCs that lose SMC characteristics and undergo transition to macrophage phenotypes also begin to proliferate and clonally expand within atherosclerotic plaques, thus exacerbating plaque growth [93].

Although the mechanisms of SMC plasticity are not well understood, it appears that pathways implicated in EndMT are also in play in the transformation of SMCs. For example, FGF and TGF β appear to regulate SMC plasticity [94]. In addition, SMC-specific conditional knockout of KLF4 resulted in reduced numbers of SMC-derived MSC- and macrophage-like cells [91•]. These findings indicate that KLF4-dependent transitions in SMC phenotype are critical in atherosclerosis. Of note, inactivation of KLF4 in vivo in mice led to a significant reduction in atherosclerotic lesion size and increased plaque stability, supporting the important role of the SMC-to-macrophage transition in atherosclerosis [91•].

The adventitia not only has been long recognized as a niche for cardiovascular and mesenchymal progenitor cells that contribute to organ homeostasis but also respond to disease stimuli (recently reviewed in [95]). Lineage tracing of Tcf21⁺ cells in the adventitia of coronary arteries revealed that vascular wall cells expressing this basic helix-loop-helix transcription factor migrate into vascular lesions of ApoE^{−/−} and Ldlr^{−/−} mice before disease initiation. While Tcf21 lineage traced cells are distributed throughout the early lesions, in mature lesions, they contribute to the formation of a subcapsular layer of cells and become associated with the fibrous cap acquiring characteristics of SMCs [96].

Sca1⁺ progenitor cells reside in the vascular adventitia [97–99]. These pluripotent cells emerge during embryogenesis and persist into adulthood. In healthy arteries of adult mice, Sca1⁺ progenitors maintain ECs and SMCs and generate vascular-like branching structures when cultured on Matrigel [100]. However, in atherosclerosis and vascular injury, Sca1⁺ cells can transition into a mesenchymal phenotype and contribute to fibrosis [97, 101–103].

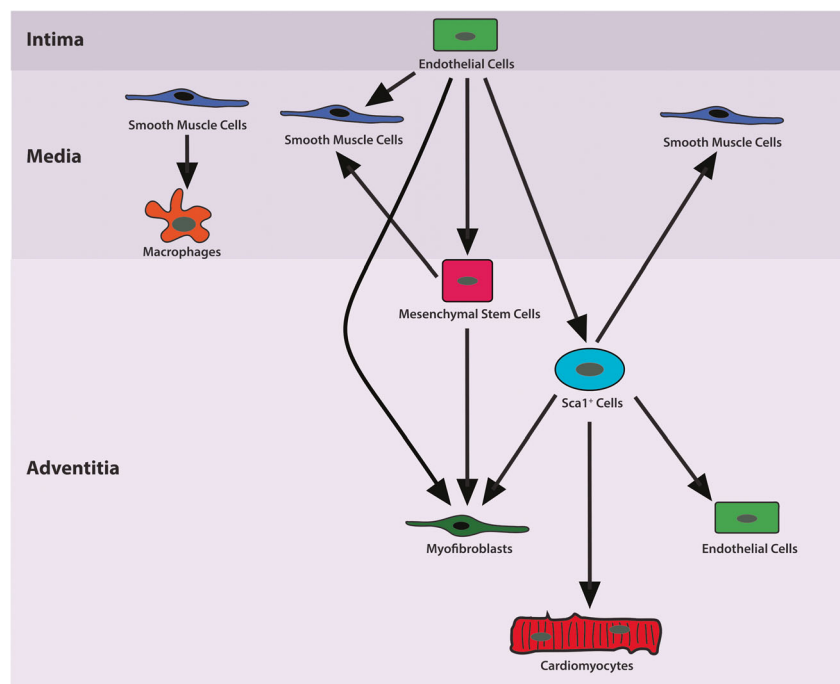
Hypertension causes arterial wall stiffening because of excessive production and deposition of collagen in the adventitia of large arteries, exacerbating blood pressure elevation and causing end-organ damage. A new study showed that most of the collagen-producing cells in the adventitia are Sca1⁺ cells [52•]. Lineage tracing and flow cytometry analyses revealed that a portion of profibrotic cells is derived from transformation of resident Sca1⁺ progenitor cells to matrix-producing cells, a second portion is derived from EndMT, and a third portion is derived from circulating bone marrow fibrocytes.

A recent publication has shown that Gli1⁺ MSCs, residing in the perivascular area in many organs, including the kidney, lung, liver, and heart, expand after injury and transform to myofibroblasts after injury, substantially contributing to organ fibrosis [104•]. Interestingly, genetic ablation of these Gli1⁺ MSCs attenuated fibrosis and improved organ function. Collectively, these lineage tracing studies suggest that progenitor cells in the adventitia of large vessels, which are necessary for vascular wall and tissue maintenance during homeostasis, become profibrotic under pathological conditions.

Conclusions

Recent discoveries have uncovered a complex picture of cellular activity around blood vessels that is important for organ homeostasis that also plays a critical role in various pathological processes. This activity depends partly on cell plasticity and the adoption of proinflammatory and profibrotic phenotypes by resident somatic cells in the intima, media, and adventitia layers, and partly on switching the differentiation potential of progenitor cells that normally regenerate organ structures during homeostasis to reparative cells that contribute to

Fig. 1 Cellular plasticity in the vascular wall. Schematic diagram of plasticity among various somatic and progenitor cell populations residing within and around blood vessels as revealed by cell lineage tracing approaches. Cellular plasticity may contribute to organ homeostasis under normal conditions, but generate proinflammatory and profibrotic cells in diseases such as atherosclerosis, myocardial infarction, heart failure, and hypertension



atherosclerotic plaque growth, interstitial fibrosis, and scar formation (Fig. 1).

The best understood process so far is EndMT that transforms endothelial cells to ECM-producing cells after acute injury or under chronic pathological conditions such as heart failure, hypertension, and atherosclerosis. It is conceivable that EndMT-derived ECM-producing cells have distinct functions than activated resident myofibroblasts, regarding their spatiotemporal contribution in the fibrotic process, the type of ECM proteins they synthesize, or their pro- and anti-inflammatory properties. On the other hand, it is also possible that the endothelial origin of ECM-producing cells is not important and that the disease environment is the primary determinant of cellular function. In this case, the multiple sources might be a means to generate the necessary cell numbers in a short period of time. It is also likely that both the origin and environment contribute to cellular heterogeneity. For example, the environment may dictate profibrotic properties during the initial stages of scar formation, but the origin may define long-term survival and specific roles in scar maintenance and neovascularization. In either case, targeting EndMT could regulate specific subpopulations of ECM-producing cells or just reduce the burden of collagen-producing cells.

Although the functional significance of cellular plasticity and fate switching in tissue repair and regeneration is poorly understood, there is evidence that these processes take place in human diseases as well. Histological analyses have identified hallmarks of EndMT in lung fibrosis, blood vessel graft stenosis, and vascular malformations [56, 105], recently reviewed in [40]. There is also evidence of SMC contribution to macrophages during atherosclerotic plaque formation in

human patients [89]. Determining the mechanisms regulating the transition of vascular and perivascular cells to proinflammatory and profibrotic cell types, and understanding the precise role of these cells in pathological conditions, should lead to new therapeutic strategies to improve clinical outcomes in patients with cardiovascular disease.

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Compliance with Ethical Standards

Conflict of Interest Antonis K. Hatzopoulos declares that he has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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