Dach1 Extends Artery Networks and Protects Against Cardiac Injury

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RATIONALE: Coronary artery disease is the leading cause of death worldwide, but there are currently no methods to stimulate artery growth or regeneration in diseased hearts. Studying how arteries are built during development could illuminate strategies for re-building these vessels during ischemic heart disease. We previously found that Dach1 deletion in mouse embryos resulted in small coronary arteries. However, it was not known whether Dach1 gain-of-function would be sufficient to increase arterial vessels and whether this could benefit injury responses.

OBJECTIVE: We investigated how Dach1 overexpression in endothelial cells affected transcription and artery differentiation, and how it influenced recovery from myocardial infarction.

METHODS AND RESULTS: Dach1 was genetically overexpressed in coronary endothelial cells in either developing or adult hearts using ApjCreER. This increased the length and number of arterial end branches expanded arteries during development, in both the heart and retina, by inducing capillary endothelial cells to differentiate and contribute to growing arteries. Single-cell RNA sequencing of endothelial cells undergoing Dach1-induced arterial specification indicated that it potentiated normal artery differentiation, rather than functioning as a master regulator of artery cell fate. Single-cell RNA sequencing also showed that normal arterial differentiation is accompanied by repression of lipid metabolism genes, which were also repressed by Dach1. In adults, Dach1 overexpression did not cause a statistically significant change artery structure before injury, but increased the number of perfused arteries in the injury zone post-myocardial infarction.

CONCLUSIONS: Our data demonstrate that increasing Dach1 is a novel method for driving artery specification and extending arterial branches, which could be explored as a means of mitigating the effects of coronary artery disease.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: cell differentiation • coronary artery disease • endothelial cells • myocardial infarction • retina
We previously demonstrated a requirement for the transcription factor Dach1 during coronary artery development. Dach1 deletion reduced coronary artery diameter by 33%. Mechanistically, Dach1 was important for artery development because it potentiated blood flow-guided EC migration into growing arteries through upregulating Cxcl12 expression. While Dach1 deletion stunted coronary artery growth by impairing EC migration, it was unclear whether Dach1 had additional roles. Coronary artery development starts with immature capillary plexus formation in heart muscle. Then, individual endothelial cells (ECs) within this plexus differentiate into arterial ECs. These preartery cells are initially intermixed throughout the plexus, but after arterial specification, migrate together to form large arteries. Whether Dach1 is also involved in these other arteriogenic processes is unknown.

Using gain-of-function experiments, we found that, in addition to its effect on migration, Dach1 also increased artery EC fate specification during embryonic and postnatal development. Single cell RNA sequencing (scRNAseq) showed that Dach1 reduced lipid transport associated genes as part of its differentiation program. Adult Dach1 overexpression mice had increased survival and heart function after myocardial infarction. Stimulating coronary artery growth can prevent, or aid in recovery after myocardial infarction. However, the pathways which regulate artery cell specification from their capillary precursors are not fully known. Here, we show that overexpression the nuclear transcriptional regulator, Dach1, can induce artery endothelial cell specification during embryonic and postnatal development. Not only do these findings describe a previously unrecognized specification pathway, but they also relate a new genetic strategy that increases coronary artery abundance far beyond what has been reported in a developmental setting. In addition, we found through single cell RNA sequencing that Dach1 mediated specification was accompanied by downregulation of lipid transport, which has not been linked to artery specification previously. Given that artery growth can affect the response to myocardial infarction, we tested the effect of Dach1 overexpression on a coronary artery ligation ischemia model in mice. This revealed that Dach1 overexpressors had greater survival and heart function after ligation. Further studying how the transcriptional and cellular changes induced by Dach1 contribute to protection from myocardial infarction could improve our ability to treat coronary artery disease in humans.

METHODS

Data Availability
All scRNA seq data including raw reads and Seurat processed data are publicly available at GEO (GSE179857).
Details of the experimental methods are available in the Data Supplement.
Please see the Major Resources Table in the Data Supplement.
For the majority of experiments, female Dach1OE/Dach1OE mice were crossed to ApjCreER males to generate Cre-/+Dach1OE/+ or Cre+;Dach1OE/+ animals used as either controls or Dach1 overexpressors respectively, upon administration of Tamoxifen. Tissue collection, fixation, immunostaining, and

Nonstandard Abbreviations and Acronyms

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<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<td>CX40</td>
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<td>DEG</td>
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What Is Known?

- Dach1 knockout mice have impaired embryonic coronary artery development.
- Growth of coronary arteries is a proposed way to protect against myocardial infarction

What New Information Does This Article Contribute?

- Overexpression of Dach1 increased artery endothelial specification during embryonic and postnatal development.
- Single cell RNA sequencing showed that Dach1 reduced lipid transport associated genes as part of its differentiation program.
- Adult Dach1 overexpression mice had increased survival and heart function after myocardial infarction

Stimulating coronary artery growth can prevent, or aid in recovery after myocardial infarction. However, the pathways which regulate artery cell specification from their capillary precursors are not fully known. Here, we show that overexpression the nuclear transcriptional regulator, Dach1, can induce artery endothelial cell specification during embryonic and postnatal development. Not only do these findings describe a previously unrecognized specification pathway, but they also relate a new genetic strategy that increases coronary artery abundance far beyond what has been reported in a developmental setting. In addition, we found through single cell RNA sequencing that Dach1 mediated specification was accompanied by downregulation of lipid transport, which has not been linked to artery specification previously. Given that artery growth can affect the response to myocardial infarction, we tested the effect of Dach1 overexpression on a coronary artery ligation ischemia model in mice. This revealed that Dach1 overexpressors had greater survival and heart function after ligation. Further studying how the transcriptional and cellular changes induced by Dach1 contribute to protection from myocardial infarction could improve our ability to treat coronary artery disease in humans.

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Using gain-of-function experiments, we found that, in addition to its effect on migration, Dach1 also increased artery EC fate specification, in both the developing heart and retina. Dach1 overexpression in plexus and capillary ECs potentiated preartery specification and lengthened artery branches. Single-cell RNA sequencing (scRNAseq) showed that Dach1 did not widely induce the expression of known artery cell fate determinants, but rather enhanced arterialization in EC subpopulations that are normally receptive to arterial specification. Finally, overexpression in adult hearts improved survival and cardiac function following myocardial infarction (MI). Our results indicate that upregulation of Dach1 could be a target for therapeutically regenerating arterial blood vessels.
imaging were performed on embryonic hearts, postnatal hearts, retinas, and adult hearts as previously described.

To prepare samples for single-cell RNA seq, embryonic hearts were enzymatically digested, and the single-cell suspension was FACS sorted to remove red blood cells and enrich for ECs. Single-cell sequencing was done using the 10X single-cell V3 platform and analysis was performed using Cell Ranger, R, and Seurat.

Coronary artery ligation experiments were done by opening the chest cavity and placing a 7-0 silk suture around the left anterior descending artery, with occlusion verified by blanching of the underlying myocardium. Transthoracic echocardiograms were then used to assess cardiac function. After fixation, ligated hearts were embedded in paraffin, sectioned, and stained with Masson’s Trichrome.

**RESULTS**

**Dach1 Overexpression Increases Artery Specification and Branching in the Heart**

Since Dach1 deletion impairs artery development, we hypothesized that increasing it would promote artery growth. Thus, we generated a transgenic mouse that would inducibly overexpress Dach1. A transgene containing the CAG promoter upstream of a Flox-Stop-Flox-Dach1-IRES-EGFP sequence was inserted into the ROSA26 locus (Figure 1A). We next crossed these with ApjCreER, which is expressed in ECs of the developing coronary capillary plexus and veins but not in differentiated arterial ECs. Cre induction with Tamoxifen resulted in excision of the transcriptional stop sequence and permanent co-expression of Dach1 and EGFP in plexus ECs. We observed high levels of Cre-dependent recombination and Dach1 expression inferred from EGFP expression (Figure 1B) and anti-Dach1 immunofluorescence (Figure 1C). Tamoxifen was given to experimental (ApjCreER;Dach1) and control (Cre-negative Dach1) mice, but we verified that neither transgene affected coronary development in the absence of Tamoxifen (data not shown). This tool was then used to explore the effects of Dach1 overexpression on artery formation.

We induced Dach1 at embryonic day (e) 13.5, when arterial EC differentiation normally begins, and harvested embryos at e15.5 (Figure 1D). At this time, single, preartery ECs that have differentiated within the immature capillary plexus have begun to coalesce into coronary arteries that can be morphologically distinguished. The effect of Dach1 was assessed by immunostaining for ECs (VE-cadherin) and the arterial EC marker CX40 (connexin 40). CX40-positive artery ECs in control hearts were in larger diameter arterial vessels and the capillary plexus as cells that have been specified as arterial but have not yet assembled into the artery, i.e., preartery cells (Figure 1E, upper). In Dach1 mutants, we observed an increase in the number of capillary plexus ECs expressing CX40 (Figure 1E, lower panels). Quantifying the area of each heart covered by CX40 positive ECs revealed an 71% increase in Dach1 OE hearts (Figure 1F). These results indicate that Dach1 overexpression stimulates preartery specification in ECs within the capillary plexus. We did not find statistically significant differences in proximal coronary artery diameters (Figure 1G), heart sizes (Figure 1H), and the number of ECs within the myocardium (Figure 1I). We concluded that Dach1 overexpression increased the abundance of arterialized ECs without grossly affecting cardiac development.

We next analyzed artery morphology 4 days post-induction at e17.5 when arteries are more mature (Figure 2A). Immunostaining for CX40 revealed that distal branches were more numerous, while diameters of proximal branches were not significantly different (Figure 2B). Additional branches expressed other artery markers (Jag1, CX37) (Figure 2A through 2C in the Data Supplement) and received blood flow as assessed by fluorescent lectin perfusions (Figure 2D and 2 E in the Data Supplement). We next determined whether the increase in artery branches resulted from Dach1 activity in capillary ECs. To test whether Dach1 has the same effects when expressed exclusively in artery ECs, we analyzed CX40CreER;Dach1 OE e15.5 embryos dosed with Tamoxifen at e13.5 (Figure 2K). In contrast to ApjCreER induction, there was no statistically significant difference in artery abundance or width in CX40CreER;Dach1 OE animals (Figure 2L through 2N). We conclude that Dach1 increases arterial branching through its activity in capillary plexus ECs.

**Dach1 Overexpression Increases Retinal Arteries Cell Autonomously**

We next used the retina to investigate whether Dach1-induced artery specification occurs in other vascular
Figure 1. Dach1OE increases artery specification during coronary artery development.

A, Dach1OE transgenic mouse line. B, Confocal image of an ApCreER;Dach1OE mouse heart (e15.5) immunostained for EGFP to assess recombination rate in the transgenic line. C, Dach1 immunostaining in Dach1OE hearts. D, Experimental strategy in E–I. E, E15.5 hearts imaged on the right lateral side show an increase in CX40 staining in Dach1OE capillaries (arrowheads). F, Quantification of the percent heart area immunostained by CX40 (n=6 control, n=4 Dach1OE; Mann-Whitney U test; Holm-Sidak adjusted P from testing 3 hypotheses). G, Quantification of primary artery diameters in e15.5 hearts (n=6 control, n=4 Dach1OE; Mann-Whitney test; Holm-Sidak adjusted P from testing 3 hypotheses). H, Erg immunostaining e15.5 heart sections. I, Quantification of endothelial density (n=3 control, n=3 Dach1OE; Mann-Whitney U test; Holm-Sidak adjusted P from testing 3 hypotheses). Scale bar=400 μM in B, E (whole heart), and H. Scale bar=200 μM in E (boxed area). Scale bar=100 μM in E highest magnification. Scale bar=50 μM in C. All data represent mean±SD. AO indicates aorta; CA, coronary artery; Cap, capillary; RV, right ventricle; and S, septum.
Figure 2. Dach1OE increases coronary artery branching.
A, Experimental strategy in B–E. B, Right lateral view of e17.5 hearts. Arrowheads indicate extra artery branches. Boxed regions show the scale of extra CX40+ vessels and the normal capillary bed morphology in Dach1OE. C–E, Quantification of the total length (C) (n=7 control, n=10 Dach1OE; Mann-Whitney U test; Holm-Sidak adjusted P from testing 3 hypotheses) and number of branch points (D) (n=7 control, n=10 Dach1OE; Mann-Whitney test; Holm-Sidak adjusted P from testing 3 hypotheses) in the CX40+ vessel network, and primary coronary artery diameters (E) in e17.5 hearts (n=7 control, n=9 Dach1OE; Mann-Whitney test; Holm-Sidak adjusted P from testing 3 hypotheses). F, Experimental strategy in G–J. G, CX40 immunostaining of the ventral surface of postnatal hearts shows increased branching in Dach1OE. H and I, Quantification of the total length (H) and branch points (I) of CX40+ vessels, and J) measurement of main coronary artery diameters in postnatal hearts (n=7 control, n=6 Dach1OE; Mann-Whitney U test; Holm-Sidak adjusted P from testing 3 hypotheses). K, Experimental strategy to generate artery specific Dach1OE expression using Cx40CreER. L, CX40 immunostaining of control and Cx40CreER;Dach1OE hearts. M and N, Quantification of CX40+ area (M) and artery diameter (N) (n=5 control, n=6 Dach1OE; Mann-Whitney U test; Holm-Sidak adjusted P from testing 2 hypotheses). Scale bar=500 μM in G. Scale bar=400 μM in B (entire heart) and L. Scale bar=200 μM in B (boxed region). All data represent mean±SD. AO indicates aorta; and CA, coronary artery.
beds. Dach1 was overexpressed in all ECs using Cdh-5CreER and a high dose of Tamoxifen at P0. VE-cadherin and CX40 expression revealed arterial morphology at P7 (Figure 3A, and Figure 1H in the Data Supplement). There was a 2.5-fold increase in the combined length of all CX40-labeled arteries in Dach1OE retinas (Figure 3B and 3C). Interestingly, arterial branches often crossed paths with veins, suggesting a breakdown of arterial-venous repulsion (Figure 3D and 3E). These data show that Dach1 is capable of extending arterial networks in a vascular bed other than the heart.

There were differences between the heart and retinal vascular beds. First, despite previous reports, we did not observe any CX40-positive cells outside arteries within the capillary plexus in either control or Dach1OE retinas, which is a hallmark of preartery cells in the heart. Artery prespecification occurs in the retina, but at the tip cell location, that is, the migrating front of the growing vasculature. Our data indicated that CX40 does not label prespecified arterial ECs in the retina. Second, Dach1OE stunted angiogenesis in retinas as demonstrated by decreased outward expansion (Figure 3B). This discordance in phenotypes likely results from the timing of Dach1OE induction in the 2 models. In the retina, Dach1 was overexpressed before the initiation of retinal angiogenesis while, in the heart, expression was induced after the coronary plexus was established. The retinal experiments also used a more widespread Cre line (Cdh5CreER) with a longer time after Tamoxifen induced recombination. Both of these factors likely contribute to a more severe angiogenic phenotype in the retina. However, the shared phenotype is increased artery branching.

We next investigated whether Dach1-induced arterialization was cell autonomous. Low-dose Tamoxifen at P0 induced mosaic Dach1OE recombination in 2.3±2.0% of retinal ECs (Figure 3F) where Dach1OE was tracked by EGFP expression (Figure 1A). ROSA26;tdTomato Cre reporter mice were used as a control. The localization of ECs to arteries, capillaries, or veins was determined at P6 and P9. Vessel subtypes were distinguished morphologically using VE-cadherin staining. At P6, the majority of tdTomato+ control cells were within capillary vessels while the remaining were distributed among arteries, veins, and tip cells, the latter of which are at the migrating front of the developing vasculature (Figure 3G). When compared with controls, the number of Dach1OE cells in arteries and at the tip position more than doubled while those in veins decreased (Figure 3H and 3I). As prespecified, arterial ECs localize to tips in the retina. The accumulation of Dach1OE ECs here is evidence that it contributes to arterial prespecification in the retina. Analyzing cellular distributions at P9 showed that Dach1OE cells no longer accumulated at tips but became even more enriched in arteries (Figure 3J through 3L). These data demonstrate that Dach1 overexpression directly causes ECs to follow a path toward arterialization in a cell autonomous fashion.

Dach1 Shifts the Endothelial Specification Trajectory Toward Arterialization

The above data and that in Chang et al contain multiple loss- and gain-of-function scenarios demonstrating that Dach1 is critical for proper coronary artery differentiation. However, its expression pattern does not suggest a simple explanation as to how. Specifically, Dach1 is expressed in most coronary ECs arising from the sinus venosus as soon as they appear on the heart. However, only a subpopulation of Dach1+ cells yields arteries. One potential explanation is our observation that protein levels (and possibly transcript levels) are heterogeneous among expressing cells, both in vivo and in vitro, and they are dramatically decreased in large arteries due to high laminar shear stress.

How does a transcription factor expressed in most coronary ECs drive differentiation of a small subpopulation into arteries? To address this question, we performed single-cell transcriptomics on Dach1OE cardiac ECs. Either tdTomato (control) or Dach1OE was induced in plexus ECs at e13.5, and cells were isolated at e15.5 by FACS followed by scRNAseq using the 10X Genomics platform (Figure 4A). Sequencing and alignment parameters served as quality controls (Figure IIA in the Data Supplement). Low-quality cells were excluded based on total number of reads, number of genes per cell, and percentage of reads aligned to mitochondrial genes (Figure IIB and IIC in the Data Supplement). To focus our analysis of coronary ECs, we also removed the endocardial cell population that lines the heart lumen (Figure IID in the Data Supplement).

Datasets were integrated and projected into 2-dimensional space using the uniform manifold approximation and projection algorithm. Unsupervised graph-based clustering partitioned ECs into 2 artery (Art1 and Art2), 1 vein, and 5 capillary clusters (Figure 4B), which were annotated based on known markers (Figure 4C). Gene expression suggested that Art1 was comprised of less mature arterial ECs whereas Art2 were more mature. Specifically, Art2 largely contained increased expression of Art1 markers with the addition of a few mature arterial EC markers such as Jag1 and Cxcl12 (Figure 4C [box], and Figure IIIA in the Data Supplement). All Art1 ECs expressed Cxcr4 but showed heterogeneous expression of Cx37 and were negative for Jag1, suggesting this cluster is a mix of arterially-skewed capillary ECs and preartery ECs (Figure IIIA in the Data Supplement).

Although distinct clusters, the uniform manifold approximation and projection topography and gene expression patterns indicated that EC subtypes exist along a continuum, rather than as completely distinct states (Figure IIIB in the Data Supplement). This was particularly evident when projecting cells on an axis of arterial-venous identity (Figure IIIC in the Data Supplement) and was consistent with previous analyses showing that
Figure 3. Dach1\textsuperscript{OE} increases retinal arterialization and promotes endothelial cell migration into arteries.

A, Dosing strategy for retina vasculature analysis. B, Retinas from Dach1\textsuperscript{OE} pups contained increased number of CX40\textsuperscript{+} vessel branches (arrowheads) and artery-vein crossing (asterisks). Right panels show insets indicated by dashed boxes. C, The total length of all CX40\textsuperscript{+} vessels per retina was greater in Dach1\textsuperscript{OE} (n=6 control, n=5 Dach1\textsuperscript{OE}; Mann-Whitney test; Holm-Sidak adjusted P from testing 2 hypotheses). D and E, Image (D, arrowheads) and quantification (E) of artery-vein crossovers in Dach1\textsuperscript{OE} retinas (n=5 control, n=5 Dach1\textsuperscript{OE}; Mann-Whitney test; Holm-Sidak adjusted P from testing 2 hypotheses). F, Experimental strategy in G–L. G–L, Images (G, H, J, and K) and quantification (I and L) of control or Dach1\textsuperscript{OE} cells in retinas from the indicated ages. Boxed regions highlight the tip and capillary cells (G and H) or artery (J and K) where there was a differential localization of control and Dach1\textsuperscript{OE} cells. (I: n=1020 control, n=767 Dach1\textsuperscript{OE}; χ\textsuperscript{2} test; L: n=1708 control, n=540 Dach1\textsuperscript{OE}; χ\textsuperscript{2} test). Scale bar=400 μM in B, G, H, J, and K (full view). Scale bar=200 μM in D, B (close up). Scale bar=100 μM in G, H, J, and K (close up). All data are mean±SD. A indicates artery; Cap, capillary; and V, vein.
Figure 4. Single-cell RNA sequencing of endothelial cells in Dach1\textsuperscript{OE} hearts. 
A, Littermate e15.5 embryos expressing ApjCreER with either Rosa26\textsuperscript{tdTomato} or Rosa26\textsuperscript{Dach1OE} were FACS sorted to isolate coronary endothelial cells for single cell sequencing. B, UMAP projections of data showed 8 endothelial cell clusters. C, The genes that define each cluster are plotted with their relative expression in each cluster. Boxed region highlights Art1 and Art2 signature genes, which are similar but with increased expression in Art2. Differential gene expression between clusters was found using the Wilcoxon Rank-sum test. D, UMAP plot showing that cells from both genotypes overlapped; Dach1\textsuperscript{OE} did not produce a new subtype. E, UMAP plots with both genotypes combined showing cell cycle stage using the CellCycleScoring function in seurat. F, Percent of cells in each cluster for both genotypes. G, Scaled expression of the Dach1\textsuperscript{OE} transgene, Dach1, and select artery markers.
brain and heart capillary ECs exist along a continuum, even in adults. Nonetheless, cells from all clusters were in both genotypes (Figure 4B and 4D), suggesting that Dach1 OE does not create a new cell identity or transcriptional state not normally present.

We also observed that cell cycle phase, inferred from the enrichment of phase-specific genes, was a major source of variability. Most capillary ECs (clusters Cap1-4) were either in S or G2/M phase (ie, cycling) while one capillary cluster (Cap5) and the artery and vein clusters were in G0/G1 (ie, noncycling; Figure 4E). In summary, scRNAseq allowed us to isolate artery, vein, and capillary (cycling and noncycling) ECs from control and Dach1 OE hearts for subsequent transcriptomic analysis.

Although all EC subtypes were in both genotypes, Dach1 OE altered the relative number of ECs within specific clusters. Dach1 OE induced a 48% increase in arterial ECs while decreasing cycling capillary ECs by 21% (Figure 4F). This corroborated our observation of increased arterial ECs while decreasing cycling capillary ECs by 21% (Figure 4F). This is consistent with the specific expansion of other clusters after adjusting for Sidak’s multiple comparisons test (Figure 5B, and Figure IVA in the Data Supplement). Consistent with ECs existing along a continuum, we sought additional evidence for this developmental trajectory. First, we determined the pattern of lineage tracing labels among different EC subtypes. These data showed that Apj, the promoter that drives Cre-mediated tdTomato (control) and EGF (Dach1 OE) expression, is only expressed in cycling cap, Cap5 G1, and Vein ECs (Figure 5G). This was supported by capillary-specific Cre recombination in ApjCreER hearts 9 hours after a Tamoxifen dose (Figure IVB and IVC in the Data Supplement). Thus, the nonartery EC subtypes initially expressed tdTomato or EGF following Cre induction at e13.5. However, after 2 days, tdTomato (control) and EGF (Dach1 OE) lineage expression was seen in arterial ECs, indicating that the artery clusters arise from Apj-positive capillary and/or vein ECs (Figure 5H). We also integrated this dataset with previously generated e12.5 and e14.5 datasets. Quantifying the ratio of cells at each time point showed that higher proportions of Art1 (less mature) precede higher proportions of Art2 (more mature) proportions (Figure 5I). Considering all of these findings, we propose that the artery trajectory includes the following steps: (1) exit of proliferating capillary ECs from the cell cycle and differentiation to a noncycling capillary subtype (Cap5 G1), (2) initial preartery/artery specification (Art1), and (3) full differentiation into mature arterial ECs (Art2) (Figure 5J).

To determine which EC subtypes are sensitive to arterialization, we calculated arterial scores, which were determined by measuring each cell’s enrichment of the genes that defined Art2 in controls (Tables I and II in the Data Supplement). Consistent with ECs existing along a continuum, control cells were distributed along the entire artery score spectrum while Dach1 OE ECs were shifted toward higher artery scores (Figure 5A). This is likely attributable to elevated artery scores in artery clusters, as artery scores were not statistically significantly different in other clusters after adjusting for Sidak’s multiple comparison test (Figure 5B, and Figure IVA in the Data Supplement). This is consistent with the specific expansion of Cx40 in Art1 as shown in Figure 4G. These data indicate that Dach1 upregulates arterial genes specifically in arterial skewed capillary, preartery, and arterial ECs.

To determine the effects of Dach1 OE on the arterial specification trajectory, we first needed to delineate this trajectory in control ECs. Trajectory analysis using monocle 3D showed that Cycling cap, Cap5 G1, Art1, and Art2 clusters were transcriptomically connected in series while veins branched off of Cap5 G1 (Figure 5C top panel). We then ordered this lineage connection by developmental stage using Cellular Trajectory Reconstruction Analysis using gene Counts and Expression. This method predicts cellular differentiation potential by calculating the expression of genes that correlate with the number of genes expressed per cell. Cellular Trajectory Reconstruction Analysis using gene Counts and Expression scored the Cycling cap cluster as the least differentiated followed by Vein, Cap5 G1, Art1, and then finally Art2 as most differentiated (Figure 5D, top and Figure 5E).

Because the above data were collected from a single time point, we sought additional evidence for this developmental trajectory. First, we determined the pattern of lineage tracing labels among different EC subtypes. These data showed that Cap5 G1, Art1, and Art2 cells were significantly more differentiated as scored by Cellular Trajectory Reconstruction Analysis using gene Counts and Expression (Figure 5D, lower and Figure 5F), while the differentiation state of Cycling cap, Vein, and Art2 ECs were not significantly different after adjusting for Bonferroni’s multiple comparison test (Figure 5D and 5F). These data suggest Dach1 is not an arterial cell fate determinant per se but potentiates arterialization in receptive cells such as Cap5 G1 and preartery cells in Art1 (Figure 5J).
Gene Expression Changes in Dach1\textsuperscript{OE}

To investigate which genes \textit{Dach1}\textsuperscript{OE} might regulate to influence arterIALIZATION, we first compared DEGs between \textit{Dach1}\textsuperscript{OE} and controls with genes that were positively or negatively enriched in control artery ECs. Each cluster had between 50 and 160 DEGs above a 0.25 log fold change (Figure 6A). Thirty-eight percentage of the Vein and 34\% of the Cycling cap DEGs that were upregulated in \textit{Dach1}\textsuperscript{OE} were on the list of 498 genes whose increase defined the Artery 2 cluster in control hearts, that is, arterial EC genes (Figure 6B, and Table I in the Data Supplement). More than 50\% of the upregulated DEGs in \textit{Dach1}\textsuperscript{OE} \textit{Cap5}\textsuperscript{G1}, Art 1, and Art 2 were also arterial EC genes (Figure 6B). In contrast, a much smaller percentage of the DEGs upregulated in \textit{Dach1}\textsuperscript{OE} were on the list of 436 that were downregulated in control arterial ECs, that is, nonarterial EC genes (Figure 6B, and Table II in the Data Supplement). When considering genes downregulated by \textit{Dach1}\textsuperscript{OE}, there were much lower percentages of overlap with arterial EC genes and nonarterial EC genes, and there was no differential pattern between the 2 types (Figure 6C). This suggests that all \textit{Dach1}\textsuperscript{OE} ECs may contain some level
**Figure 6. Specific gene expression changes in Dach1OE.**

A. The number of differentially expressed genes (DEGs) when separately comparing control and Dach1OE cells from each cluster. B and C. Genes that are either positively or negatively enriched in control artery clusters and termed artery genes and nonartery genes, respectively. DEGs that were either up- or downregulated by Dach1OE in each cluster were then compared with these lists. Upregulated DEGs in Dach1OE have strong overlap with artery genes (B) while downregulated DEGs in Dach1OE have less overlap with nonartery genes (C). Venn diagrams showing overlap of artery (D) and nonartery (E) genes with DEGs either up- or downregulated by Dach1OE in noncycling capillary. F. Cell scores generated for select lipid pathways (see methods) showed a reduction in Dach1OE Cap551 (2-way ANOVA, P represents genotype factor). G-J. Validation of decreased Fabp4 in Dach1OE cells using immunofluorescence on hearts at e15.5 (n=4 per group, Kruskal-Wallis test). I and J, Adult stages (n=4 control, n=4 Dach1OE, Mann-Whitney test). K-L. DEGs shared between endothelial cells experiencing Dach1 overexpression in either developing mouse hearts (Dach1OE) or primary cell culture (Dach1-HCAECs) (hypergeometric test). L. Overlap between mouse Dach1OE DEGs and genes that are positively or negatively correlated with endogenous Dach1 in scRNAseq data from control hearts (hypergeometric test). Scale bar=50 μM in G. Scale bar=100 μM in I. Red bar=mean in F, data represent mean±SD in H and J.
of arterial priming through the induction of genes not previously correlated with artery fate, and that there is a greater induction of artery genes rather than repression of nonartery genes.

Since lineage data suggested Cap5 cells were the direct artery progenitors (Figure 5C through 5H) and differentiation state of this cluster was affected by Dach1OE (Figure 5F), the genes changed in this subpopulation could influence differentiation. Upregulated DEGs included genes that stimulate artery differentiation such as Gja4 (CX37)22 and Sox7,23 or artery remodeling such as Cxcl12 (Figure 6D). Unexpectedly, downregulated DEGs included several lipid transport and metabolism genes (Figure 6E). Thus, we investigated additional lipid metabolism and signaling pathways and found that genes associated with lipid transport, lipid oxidation, and eicosanoid metabolism were significantly downregulated by Dach1OE (Figure 6F; and Figure IVD through IVG in the Data Supplement). The change in one of these, Fabp4, was validated at the protein level in both embryonic and adult hearts (Figure 6G through 6J). Although not typically linked to artery development, these data suggest suppression of lipid metabolism might be involved.

Since arterial specification is linked to cell cycle exit,10,22 we noted that Cdkn1c expression, a cell cycle inhibitor, was upregulated by Dach1OE (Figure 6D). EdU incorporation experiments marked the number of cells entering S-phase to assess whether Dach1 decreased cell cycling. Analyses focused on nonarterial regions to avoid confounding results because of cell cycle exit being linked to arterial differentiation. There was no statistically significantly difference in EdU incorporation in Dach1OE vessels where arterial differentiation does not occur (Figure IVH in the Data Supplement). It was decreased within intramyocardial regions, but this could be due to the increased arterial differentiation at that location (Figure IVI in the Data Supplement). EdU incorporation was not significantly different in the retina (Figure IVJ in the Data Supplement). Thus, although not affecting S-phase entry in all cells, upregulation of Cdkn1c could potentiate cell cycle arrest in the presence of other artery differentiation signals. Dach1OE scRNAseq DEG lists were cross-referenced with a previously generated bulk RNAseq dataset from cultured human coronary artery ECs overexpressing Dach1 (Dach1-HCAECs).8 We found that 26.4% of the genes upregulated in Dach1OE mouse coronary ECs were also upregulated in Dach1-HCAECs, and 30.7% of downregulated genes overlapped with those downregulated in cultured cells (Figure 6K). Notable genes regulated in both systems were Cxcl12, Aqp1, Cdkn1c, and lipid transport genes (Figure 6K). This analysis identified genes regulated by Dach1OE in both mouse ECs in vivo and cultured human ECs.

As mentioned above, although Dach1 is expressed in most coronary ECs, protein levels vary from cell-to-cell,8 which in scRNAseq data results in different levels of mRNA and includes cells with none detected at all (Figure 4G). Given the protein expression patterns and detection limits of scRNAseq, the lack of transcripts likely reflects low expression instead of absence. This allowed us to investigate whether the genes induced by Dach1OE reflect the genes that are endogenously regulated by higher levels of Dach1. Cells from control hearts only were used to correlate presence of Dach1 mRNA in scRNAseq data to the expression of all other genes. Identifying the top 500 genes positively and negatively correlated with endogenous Dach1 and comparing those to Dach1OE DEGs revealed strong overlaps (Figure 6L).

Prominent on these lists were arterial EC and nonarterial EC genes, such as Aqp1 and lipid transporters, respectively. This supports the idea that overexpressing Dach1 affects similar transcriptional programs as endogenous Dach1, and that this transcription factor regulates artery and lipid transport genes in multiple settings.

**Dach1 Overexpression Supports Recovery Post-MI**

We next investigated whether Dach1 overexpression improved outcomes following MI. Left anterior descending coronary arteries were permanently ligated in 12-week-old adult mice with ApjCreER-induced Dach1 overexpression in capillary and venous ECs for 6 weeks before injury (Figure VA in the Data Supplement). Quantification showed Dach1 overexpression in 64.5±14% of coronary ECs (Figure VB in the Data Supplement).

MI resulted in 43.3% survival for control animals (Figure 7A). Most fatalities occurred within 10 days post-MI while Dach1OE mice exhibited a 90.6% survival rate (Figure 7A). Echocardiography revealed that Dach1OE mice had higher ejection fractions than controls over the course of the study (Figure 7B). A control experiment using the same genotypes without Tamoxifen showed that this rescue effect was not due to the ApjCreER transgene (data not shown).

Histology at 4 weeks revealed reduced fibrosis in Dach1OE (Figure 7C and 7D). In 92% of WT mice, fibrotic scars extended the full thickness of the myocardium, whereas this pattern was seen in only 59% of Dach1OE hearts (Figure 7E). In hearts without full thickness scars, fibrosis was limited to mid-myocardial regions (Figure 7C and 7E). Mice with no scar were excluded from analysis due to technical failure. Measuring myocardial thickness at sequential points around the entire heart revealed an increased thickness in Dach1OE left ventricles (Figure 7F).

We investigated whether improvements were due to arterial changes. Measuring the percent area stained by the artery marker smooth muscle actin 6 weeks after Tamoxifen but before injury did not reveal a statistically significant difference between control and Dach1OE (Figure 7G), which was consistent with qualitative analyses.
Figure 7. Dach1 overexpression promotes survival after myocardial infarction. 

A, Survival curve during 4 wks post-MI (n=30 control, n=32 Dach1OE; Log-rank test). 
B, Percent ejection fraction at the indicated time points (n=29 control, n=30 Dach1OE; above graph: mixed effect model above (P represents genotype factor); individual comparisons: Holm-Sidak adjusted). 
C, Hematoxylin & Eosin staining on representative hearts. Arrow highlight an example of a mid-myocardial scar. 
D, Percent of total myocardium stained with Masson’s Trichrome in sections from 3 levels posterior to the ligation (n=13 control, n=29 Dach1OE; Mixed effect model, P represents genotype factor). 
E, Quantification of scarring pattern. 
F, The width of the myocardium at 360 angles around the heart. In the left ventricle where the infarct was induced, Dach1OE better preserved myocardial thickness when compared with controls. Lines are averages of each group while shading indicates SD (n=13 control and n=29 Dach1OE; Mixed effect model, P represents genotype factor). 
G and H, Smooth muscle actin (SMA) staining in sections from uninjured (G) (n=5 control, n=5 Dach1OE; Mann-Whitney U test) and post-MI hearts (H) (n=12 control, n=29 Dach1OE; 1-way ANOVA) with quantification of SMA density. 
I and J, Isolectin perfusion 2 h (n=5 control, n=4 Dach1OE; Mann-Whitney U test) or 5 d (n=5 control, n=7 Dach1OE; Mann-Whitney U test) after MI, asterisks indicate stitch location. Arrows show perfused vessels connecting from perfused regions into the infarct zone in Dach1OE. 

Scale bar=1 mm in C, I, J. Scale bar=500 μm in G. Scale bar=250 in H. Error bars show mean±SD.
of whole mount images (Figure VC in the Data Supplement). Four weeks after injury, 
\(Dach^{10E}\) hearts with mid-myocardial scars had a higher smooth muscle actin+ areas, indicating increased arterialization in hearts with the best recovery (Figure 7H). This effect did not seem to be driven by the hearts with mid-myocardial scars merely having more intact myocardium since, unexpectedly, scarred regions still contained many arteries (Figure 7H). To compare infarct sizes, fluorescent lectin was injected into ventricles post-mortem to perfuse the coronary system either at 2 hours or 5 days after coronary ligation. At 2 hours, infarct sizes were not significantly different (Figure 7I). Five days post-MI, \(Dach^{10E}\) hearts contained isolated perfused vessels running from the border zone into infarcted tissue in 60% of the samples (Figure 7J). Whether these partially perfused regions would increase with longer perfusion techniques or whether they would be enough to explain the observed myocardial protection will be the subject of future studies. Together, these patterns indicate that adult overexpression of \(Dach1\) does not change pre-existing artery structure but increases arteries and affects perfusion to the infarcted area in the days after injury.

To observe whether more dramatic effects on artery structure could be obtained by over expressing \(Dach1\) from earlier stages, \(ApicCre;Dach^{10E}\) mice were dosed with Tamoxifen at e13.5 and kept until adulthood. All treated mice survived, but with enlarged hearts (Figure IF in the Data Supplement). Visualization of adult artery trees indicated that the extra artery branching phenotype was either lost or less extensive in adults (Figure IG in the Data Supplement).

In addition to arterial expansion after injury, unrelated gene expression changes could also underlie the protective injury response. \(Dach1\) suppressed lipid transport genes, and recent studies showed that decreasing the heart’s utilization of fatty acids promotes adult cardiomyocyte proliferation and heart regeneration. It is possible that changes in endothelial lipid transport to cardiomyocytes could similarly contribute to improved outcomes after MI. Blood profiles of uninjured mice revealed baseline decreases in total cholesterol and HDL cholesterol in the blood of \(Dach^{10E}\) mice, suggesting misregulation of systemic lipid pathways (Figure VD and VE in the Data Supplement). It will be interesting to ascertain any functional consequences in future studies.

**DISCUSSION**

Coronary artery ECs differentiate from capillaries; however, transcriptional regulators of this transition remain incompletely understood. We found that overexpression of \(Dach1\) drove ectopic arterial EC specification in coronary capillaries. Extra arterial ECs contributed to artery remodeling to create longer, more branched arterial networks, which was also detected in the retina. ScRNAseq revealed that \(Dach^{10E}\) suppressed lipid transport genes in all ECs but upregulated arterial genes specifically in capillaries on the arterial side of the artery-vein vein continuum and in preartery and arterial populations. Finally, \(Dach1\) overexpression in adults improved survival and heart function after MI, which was accompanied by an increase in arteries and perfusion that occurred only in the days after injury.

Mosaically expressing \(Dach^{10E}\) in retinal ECs resulted in preartery and arterial populations. Finally, \(Dach1\) overexpression in adults improved survival and heart function after MI, which was accompanied by an increase in arteries and perfusion that occurred only in the days after injury.

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Disclosures

None.

Supplemental Materials

Expanded Materials & Methods

Data Supplement Figures I–V

Data Supplement Tables I and II

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