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Temporal Regulation of Venous Extracellular Matrix Components during Arteriovenous Fistula Maturation

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Abstract

Purpose—The venous limb of arteriovenous fistulae (AVF) adapts to the arterial environment by dilation and wall thickening; however the temporal regulation of the expression of extracellular matrix (ECM) components in the venous limb of the maturing AVF has not been well characterized. We used a murine model of AVF maturation that recapitulates human AVF maturation to determine the temporal pattern of expression of these ECM components.

Methods—Aortocaval fistulae were created in C57BL/6J mice and the venous limb was analyzed on post-operative days 1, 3, 7, 21, and 42. A gene microarray analysis was performed on day 7; results were confirmed by qPCR, histology, and immunohistochemistry. Proteases, protease-inhibitors, collagens, glycoproteins and other non-collagenous proteins were characterized.

Results—The maturing AVF has increased expression of many ECM components, including increased collagen and elastin. Matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinase 1 (TIMP1) showed increased mRNA and protein expression during the first 7 days of maturation. Increased collagen and elastin expression was also significant at day 7. Expression of structural proteins was increased later during AVF maturation. Osteopontin (OPN) expression was increased at day 1 and sustained during AVF maturation.

Conclusion—During AVF maturation there is significantly increased expression of ECM components, each of which shows distinct temporal patterns during AVF maturation. Increased expression of regulatory proteins such as MMP and TIMP precedes increased expression of structural proteins such as collagen and elastin, potentially mediating a controlled pattern of ECM degradation and vessel remodeling without structural failure.

Keywords

Arteriovenous Fistula; Extracellular Matrix; Vascular Fistula

Introduction

The arteriovenous fistula (AVF) is the most frequently performed vascular access for hemodialysis in patients with end-stage renal disease.(1,2) Similar to the process of vein graft adaptation to the arterial environment, the AVF also adapts to the arterial environment, with maturation of both the arterial inflow limb as well as the venous outflow limb of the AVF. Maturation requires vessel wall dilatation and thickening and is thought to be an adaptive process to the increase in pressure, shear stress, and oxygen tension from the arterial inflow that is no longer attenuated by the resistive forces of the arterioles and capillary beds. Differing from vein grafts, which are veins removed from their anatomic position and interposed within the arterial circulation, the venous limb of the AVF remains in situ but is surgically joined to a neighboring artery and thereby directly exposed to the properties of the arterial circulation.(3)

Previous studies of venous adaptation to the arterial environment typically report findings in vein grafts, with fewer studies examining AVF. Kwei et al used a jugular-to-carotid AVF in mice to demonstrate that the venous limb of the AVF retains endothelial coverage and underlying smooth muscle cells proliferate.(4) Castier et al also used a mouse jugular-to-carotid AVF model to study anastomotic neointimal hyperplasia, similar to that found in human AVFs, and found that this hyperplasia was a result of local stimulatory factors and not from circulating progenitor cells.(5) However there remains a relative paucity of data describing the unique venous adaptation to the arterial environment in the AVF.

We recently described a mouse aortocaval fistula model that mimics human AVF maturation, with a distinct maturation phase during which approximately two-thirds of AVF successfully mature and one-third of AVF fail to mature.(6) We use this model to examine the regulation of ECM components during AVF maturation. We hypothesized that during AVF maturation, regulatory and structural components of the ECM have organized temporal expression patterns.

Materials and Methods

Arteriovenous Fistula Creation

Aortocaval fistulae were created as previously described. (6,7) In brief, AVF were created in C57BL/6J male mice at age 10 weeks (20–30g) using a 25 gauge needle to puncture the lateral wall of the aorta, through and through, entering the inferior vena cava caudal to the renal vessels. The lateral puncture site of the aorta was sealed with gentle manual compression of the surrounding tissue until hemostasis achieved, with care taken not to compress the fistula between the aorta and the inferior vena cava. The presence of the AVF was confirmed by direct visualization of pulsatile arterial blood flow in the IVC as well as serial Doppler ultrasound starting on day 1. Each AVF was accompanied by a paired mouse

that underwent a sham procedure consisting of midline laparotomy without the AVF creation.

The venous limb of the AVF was analyzed at predetermined time points by carefully removing the abdominal IVC from the point of the fistula cephalad to the hepatic confluence. Samples were taken from baseline (pre-operative) mice, as well as post-operative day 1, 3, 7, 21, and 42, each with a paired sham IVC control. Prior to harvest, ultrasound interrogation was performed to determine AVF patency. Occluded AVF at any time point were excluded.(6)

Gene Expression Microarray Analysis

Gene expression in the venous limb of the AVF was compared to that in the IVC of sham mice (day 7; n=4). RNA was isolated from pooled samples and analyzed for whole genome gene expression using the Mouse Gene 1.0 ST array (Cat. No. 901168, Affymetrix; Santa Clara, CA). Samples were hybridized to arrays for 16 hours at 45 °C. Significant changes between AVF and sham veins in gene expression were determined using a false discovery rate <0.05 and a fold change >2.0 or <-2.0. Significantly increased or decreased expression of gene pathways were analyzed using principle component analysis and pathway enrichment analysis (MetaCore, Thomson Reuters, New York, NY).

RNA Extraction & Real-Time Polymerase Chain Reaction (RT-PCR)

The venous limb of the fistula (specimens) or the IVC (control) was removed with care taken to avoid surrounding arterial and connective tissue and total RNA isolated using RNeasy Mini Kit with digested DNase I (Qiagen, Valencia, CA). RNA quality was confirmed using the 260/280nm ratio (Nanodrop; Thermo Scientific, Wilmington, DE). Reverse transcription was performed using SuperScript III First-Strand Synthesis Supermix (Invitrogen; Life Technologies, Carlsbad, CA). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Samples were amplified for 35 cycles using the iQ5 Real-Time PCR Detection System (Bio-Rad). Correct target amplification and exclusion of nonspecific amplification was confirmed by 1.5% agarose gel electrophoresis; primer efficiencies were determined by melt curve analysis. Primers used are as listed in Table 1. All samples were normalized by GAPDH RNA amplification.

Histology

Venous limbs of AVF and IVC were extracted *en bloc* after circulatory flushing with PBS followed by 10% formalin. The tissue block was then embedded in paraffin and cut in 5-µm cross-sections. Hematoxylin & eosin (H&E), Masson's Trichrome, and elastic van Gieson (EVG) staining were performed on samples from preoperative as well as day 1 through day 42 AVF.

Antibodies

A mouse monoclonal antibody directed against mouse MMP-2 (Clone: 6E3F8) (ab86607) and rabbit polyclonal antibodies directed against mouse MMP-9 (ab38898), TIMP-1 (ab38978), and osteopontin (ab8448) were purchased from Abcam (Cambridge, MA).

Antibodies against mouse collagen type I, III and fibronectin were raised in rabbits and purified as described elsewhere.(8,9)

Immunohistochemistry

The venous limb of the AVF was analyzed approximately 100 microns cranial to the fistula, i.e. between the fistula and renal vessels. Immunohistochemistry was performed using the Dako EnVision™ + Dual Link System-HRP (Dako; Carpinteria, CA). For collagen type I, III, and fibronectin, sections were pre-treated with 3.0% hyaluronidase (bovine testicular origin, type I-S; Sigma-Aldrich Co, St. Louis, MO) in PBS (pH 7.4) for 30 min at 37°C. For MMP-2, MMP-9, and TIMP-1, sections were heated in citric acid buffer (pH 6.0) at 100°C for 10 min using Lab Vision PT Module (Thermo Scientific; Kalamazoo, MI). The sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity and incubated with 5% normal goat serum in PBS (pH 7.4) containing 0.05% Triton X-100 (T-PBS) for 1 h at room temperature to block non-specific protein binding sites. Sections were then incubated at 4°C with the primary antibodies diluted at 1:200 (anti-collagen type I, III, and fibronectin), 1:300 (anti-MMP-2), and 1:500 (anti-MMP-9, TIMP-1, and OPN) in T-PBS. After overnight incubation, the sections were incubated with EnVision reagents for 1 h at room temperature and treated with Dako Liquid DAB+ Substrate Chromogen System (Dako) to visualize the reaction products. Finally, the sections were counterstained with Dako Mayer's Hematoxylin (Lillie's Modification) Histological Staining Reagent (Dako).

Relative quantification of histological and IHC staining was performed (MetaMorph, Molecular Devices, LLC, Sunnyvale, CA). Each venous limb of AVF sample was compared to respective sham vein sample of the same post-operative day where applicable and all samples were compared to controls stained simultaneously.

Statistical Analysis

All data was analyzed using Prism 6 software (GraphPad Software, Inc, La Jolla, CA). Comparison of AVF samples to paired sham samples as well as baseline pre-operative samples were made using paired t-test or one-way ANOVA with post-hoc analysis using Dunnett's multiple comparisons test where applicable. P values of < .05 were considered significant.

Microarray analysis was performed using hierarchical clustering analysis as well as principle component analysis and pathway enrichment analysis (MetaCore). Significant changes in gene expression of AVF compared to paired sham IVC were determined using a false discovery rate <0.05 and a fold change >2.0 or <-2.0.

Results

Compared to preoperative veins, histological analysis of AVF revealed temporal changes in the vessel wall characteristics during the forty-two day study period (Figure 1A–C). The fistula thickened over time, compared to the preoperative vein, consistent with AVF maturation (Figure 1D). The number of cells in the venous limb increased during AVF maturation compared to control veins (Figure 1E). Trichrome staining showed increased

collagen and matrix deposition during AVF maturation (Figures 1F). Similarly, elastin deposition was increased compared to control veins later during AVF maturation (Figure 1G).

Since day 7 was associated with early AVF thickening (Figure 1D), gene expression changes in the day 7 venous limb were compared to sham veins using microarray analysis. There were 1196 genes with significantly altered expression; 654 genes were increased and 542 genes decreased by 2-fold or more in the AVF compared to veins. The genes with the most highly increased and decreased expression are listed in Table 2. Pathway enrichment analysis showed 38 significantly regulated pathways, with metabolism and cell cycle pathways being the pathways with the most highly increased expression (Table 3).

Since AVF maturation is characterized by wall thickening and deposition of ECM (Figure 1), we analyzed the pathway regulating synthesis and secretion of ECM components during AVF maturation; there was significantly increased expression of 14 of the 50 genes included in this pathway (Table 4). We hypothesized that factors regulating ECM synthesis, secretion, or deposition would have earlier temporal expression compared to later expression of structural components of the ECM. Accordingly, ECM components were grouped into 4 categories for analysis: proteases, protease inhibitors, collagens, and glycoproteins/non-collagenous proteins (Table 5). The temporal expression of select members of each of these 4 groups was confirmed by PCR during the 42-day course of AVF maturation and summarized in Table 6. Characteristic members of each of these 4 groups were analyzed in additional detail.

The temporal regulation of mRNA expression of several MMP is summarized in Table 6. Expression of MMP-2 mRNA in the AVF was significantly increased, compared to control veins, at day 7 (Figure 2A). IHC showed increased MMP-2 immunoreactivity (Figure 2B) in both the arterial and venous limbs of the AVF (Figure 2C), with increased MMP-2 staining in the endothelium of the venous limb compared to control veins (Figure 2D). In contrast, MMP-9 mRNA expression was increased maximally on day 1 compared to control veins (Figure 3A). IHC showed increased MMP-9 immunoreactivity (Figure 3B) in the venous limb (Figure 3C) compared to control veins (Figure 3D).

The temporal regulation of mRNA expression of the TIMP family is summarized in Table 6. Expression of TIMP1 was increased at day 1 and remained elevated until day 21 compared to control veins (Figure 4A). IHC showed increased TIMP1 immunoreactivity (Figure 4B) diffusely at day 1 (Figure 4C) compared to control veins (Figure 4D). TIMP2, TIMP3, and TIMP4 expression remained unchanged at early times and increased at day 21, with 7-fold, 6-fold, and 2-fold increases, respectively, compared to control veins (Table 6).

Collagens showed increased mRNA expression at day 7 (Table 6). Both collagen I and collagen III had increased expression compared to control veins (Figures 5A, 6A), although collagen III showed an earlier increase in expression (Figure 6A). IHC showed increased interstitial deposition of both collagen I and collagen III (Figures 5B, 6B). Collagen I deposition was found within the layers of the remodeling vein (Figure 5C), whereas collagen III showed strong staining in the adventitia (Figure 6C).

Select non-collagenous proteins were also analyzed as summarized in Table 6. Osteopontin (OPN) has been previously shown to have increased expression during early AVF maturation (10); OPN mRNA expression was increased throughout AVF maturation (Figure 7A). A significant increase immediately on day 1, with over 40-fold increase in expression relative to control veins, was confirmed with IHC (Figure 7B–D). Fibronectin (Fn) mRNA expression was increased on day 21 (Figure 8A); there was a 3-fold increase in Fn protein (Figure 8B) in AVF compared with control veins (Figure 8C–D). Expression of elastin mRNA was increased on days 3 and 7 (Table 6), preceding deposition of elastin into the AVF (Figure 1G).

Discussion

The dynamic process of ECM expression, synthesis, secretion, and deposition during AVF venous limb maturation occurs in distinct temporal phases (Figure 9). Initial ECM degradation occurs early after AVF creation, coincident with early increased expression of MMP and TIMP1. By day 7 there is increased expression of many collagen subunits as well as changing patterns of MMP expression. By day 21, a later phase is characterized by reduced MMP expression and increased expression of larger structural and non-collagenous matrix proteins. These results show distinct temporal regulation of the ECM components during AVF maturation.

The microarray analysis comparing mRNA expression in the AVF with sham IVC was performed on day 7 after surgery. This time point is consistent with generally accepted maximal vessel proliferation during venous adaptation to the arterial circulation. Zwolak et al described maximal endothelial and smooth muscle cell replication at day 7 in a rabbit vein graft model.(11) Castier et al reported that those smooth muscle cells accumulated from the stimulated resident cells and not from circulating progenitor cells.(5) The pathways generated from this data (Table 3) are consistent with these findings as the metabolic and cell proliferation pathways were the pathways with the most increased activity at this time point. Our finding of increased cell numbers after day 7 (Figure 1E) likely reflects both cell proliferation as well as cell migration into the AVF.

Increased MMP-2 in the serum of adult human patients have been correlated with AVF maturation.(12) We demonstrate that, during the early phase of ECM breakdown, there is increased expression of several MMP (Table 6; Figure 2). This increased MMP expression may be required for degradation of the vessel structure to accommodate the structural changes associated with maturation but these changes must occur without wall weakening and structural failure. Misra et al have demonstrated a 2-fold increase in MMP-2 at day 14 and over 50% increase in MMP-9 at day 28 in the venous stenosis in a rat AVF model, suggesting that MMP activity later in AVF maturation may be associated with AVF failure due to stenosis formation.(13) Increased MMP-2 and MMP-9 expression is implicated in vessel wall dilation and structural weakening in both human and murine abdominal aortic aneurysm (AAA) formation.(14,15) In addition, increased MMP-9 expression in both the aortic wall and serum is associated with ruptured AAA in human patients.(16,17) The stimulus for this early increase in MMP expression in our study may be secondary to vessel injury from the AVF surgery as well as a normal regulatory maturation process.(18,19)

Expression of MMP-14 was increased on day 7 by over 5-fold in the microarray analysis (Table 4). MMP-14 is a membrane-bound protein regulator of other proteins involved in matrix modulation, cell migration, and signaling between the matrix and cells. MMP-14 activates pro-MMP-2(20), degrades ECM components including collagens and fibronectin(21), and promotes cell migration via heterodimerization with membrane receptor CD44.(22) Our data suggests an orderly expression pattern of MMP during AVF maturation that is probably essential for vessel remodeling without wall weakening and degradation, whereas large increases in these proteins may be associated with vessel wall weakening and rupture.

The TIMP family is a group of endogenous specific inhibitors of the MMP. TIMP 1–3 inhibit smooth muscle cell proliferation in human saphenous veins and vein grafts.(23–25) However TIMP are associated with both AVF maturation as well as AVF failure; TIMP-2 and –4 are associated with AVF maturation (12,26) and TIMP-1 and –2 are associated with AVF failure (27) in human and murine models, respectively. In our mouse model, TIMP1 is the most highly expressed TIMP, with sustained increased expression from days 1–21. TIMP1 is thought to be the predominant regulator of the pathological effects of MMP in aneurysmal disease, with an increased MMP:TIMP1 expression ratio associated with aneurysm formation.(28,29) TIMP1 knockout mice develop larger aneurysms than controls. (30) Our data suggests that TIMP1 may facilitate venous dilatation and AVF remodeling.

We report increased collagen and other structural protein expression and deposition at later time points during AVF maturation. Increased early elastin and fibrillin-1 mRNA expression (Table 6) followed by later elastin fragment deposition (Figure 1G) demonstrates orderly venous remodeling that retains structural integrity during venous adaptation to the arterial environment. Although elastin is usually stable with a very long half-life, increased elastin and/or fibrillin-1 expression during AVF adaptation may be a marker of venous “arterialization”, e.g. loss of venous identity during venous adaptation to the arterial environment (31). Many of the large matricellular proteins are also actively involved in the regulation of vessel changes in addition to their roles as scaffolds for vessel remodeling. For example, an internal fragment of collagen XVIII is endostatin, a potent angiogenic inhibitor(32); fibronectin regulates smooth muscle cells during vascular wall remodeling(33,34); and thrombospondin-2, a potent anti-angiogenic component of the ECM, modulates MMP-2 and MMP-9 activity in endothelial cell ECM(35).

Several previous reports showing increased MMP and TIMP expression in AVF have focused on the failing fistula(27,36–38); we report the temporal expression sequence that occurs during successful AVF maturation. Although Kwei et al described early AVF maturation, it is not clear whether the AVF matured successfully or failed subsequent to the early time points examined; in addition, they focused on inflammation and expression of E-selectin and VCAM-1.(4) This group also reported a microarray analysis of the AVF at day 7, and described OPN as the gene with highest expression in the remodeling AVF.(10) Similar to the findings of Abeles et al, the matrix of the AVF is one of the most significantly altered pathways in microarray analysis of AVF maturation.(10) We report the changes in the venous limb and the ECM over the course of AVF maturation, a notable limitation of their study.

We also report a significant increase in osteopontin that persists throughout AVF maturation. Osteopontin, an ECM-modulating protein that is involved in neointimal hyperplasia formation(39), increases during venous remodeling. (40,41) Liaw et al found osteopontin essential to neointima formation in a rat carotid artery injury model suggesting its function as an initiator of general vascular remodeling.(42) Osteopontin also modulates MMP-2 and MMP-14 suggesting direct involvement of these genes in the remodeling pathway. (39,43) In addition, Zohar et al described the co-localization of osteopontin and CD44 (a known receptor for hyaluronan) as a functional complex supporting hyaluronan-mediated mechanisms of matrix changes.(44) While the role of osteopontin is not specific to venous remodeling, our data supports a major role for osteopontin in AVF maturation.

Temporal regulation of gene expression is a common paradigm. For example, the Hox genes are expressed along a structural head-to-tail axis (spatial collinearity) as well as in temporal stages (temporal collinearity) in the developing vertebrate.(45) The temporal regulation of gene expression during AVF maturation is poorly described in previous reports, with many reports analyzing only a single time point. Our finding of the expression of regulatory components prior to the structural components allows focus on this earlier time point to find appropriate control points for therapeutic manipulation.

Conclusion

We demonstrate that the expression of ECM components during AVF maturation is a dynamic process that occurs in distinct temporal phases. We believe that these distinct phases allow regulated changes in ECM components to allow venous adaptation to the arterial environment without loss of the vessel's structural integrity.

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Figure 1.

Histology of AVF maturation. **A)** Representative photomicrographs stained with hematoxylin and eosin (H&E), days 0–42. Scale bar, 40 μm . **B)** Representative photomicrographs stained with trichrome stain, days 0–42. Scale bar, 40 μm . **C)** Representative photomicrographs stained with van Geison stain, days 0–42. Yellow arrows indicate positive elastin staining. Scale bar, 40 μm . **D)** Bar graph showing wall thickness of venous limb of AVF, days 1–42. $n=3$. $P = .0001$. **E)** Bar graph showing mean number of nuclei per high power field, normalized to day 0. $n=3$. $P < .0001$. **F)** Bar graph showing relative quantification of collagen staining, normalized to day 0. $n=3$. $P = .0036$. **G)** Bar graph showing relative quantification of elastin staining, normalized to day 0. $n=3$. $P = .002$. A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF. A.U., arbitrary units

Figure 2.

Matrix metalloproteinase 2 (MMP-2) expression during AVF maturation. **A)** Bar graph showing mRNA transcripts of MMP-2 in the AVF venous limb relative to sham vein (normalized to day 0; all samples normalized to GAPDH), days 0–42. $n=8$. $P = <.0001$. **B)** Bar graph showing relative quantification of positive immunostaining of MMP-2 protein in the AVF venous limb (normalized to sham vein), day 7. $n=3$. $P = .0006$. **C)** Representative photomicrograph of immunohistochemistry staining of MMP-2 protein in AVF venous limb at day 7. Scale bar, 40 μ m. **D)** Representative photomicrograph of immunohistochemistry staining of MMP-2 protein in sham vein, day 7. Scale bar, 40 μ m.

A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF

Figure 3.

Matrix metalloproteinase 9 (MMP-9) expression during AVF maturation. **A)** Bar graph showing mRNA transcripts of MMP-9 in the AVF venous limb relative to sham vein (normalized to day 0; all samples normalized to GAPDH), days 0–42. $n=8$. $P = <.0001$. **B)** Bar graph showing relative quantification of positive immunostaining of MMP-9 protein in the AVF venous limb (normalized to sham vein), day 1. $n=3$. $P = .21$ **C)** Representative photomicrograph of immunohistochemistry staining of MMP-9 protein in AVF venous limb, day 1. Scale bar, 40 μ m. **D)** Representative photomicrograph of immunohistochemistry staining of MMP-9 protein in sham vein, day 1. Scale bar, 40 μ m.

A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF

Figure 4.

Tissue inhibitor of metalloproteinase 1 (TIMP1) expression during AVF maturation. **A)** Bar graph showing mRNA transcripts of TIMP1 in the AVF venous limb relative to sham vein (normalized to day 0; all samples normalized to GAPDH), days 0–42. $n=8$. $P < .0001$. **B)** Bar graph showing relative quantification of positive immunostaining of TIMP1 protein in the AVF venous limb (normalized to sham vein), day 1. $n=3$. $P = .002$. **C)** Representative photomicrograph of immunohistochemistry staining of TIMP1 protein in AVF venous limb, day 1. Scale bar, 40 μ m. **D)** Representative photomicrograph of immunohistochemistry staining of TIMP1 protein in sham vein, day 1. Scale bar, 40 μ m.

A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF

Figure 5.

Collagen I (COL I) expression during AVF maturation. **A)** Bar graph showing mRNA transcripts of COL I in the AVF venous limb relative to sham vein (normalized to day 0; all samples normalized to GAPDH), days 0–42. $n=8$. $P < .0001$. **B)** Bar graph showing relative quantification of positive immunostaining of COL I protein in the AVF venous limb (normalized to sham vein), day 1. $n=3$. $P = .003$. **C)** Representative photomicrograph of immunohistochemistry staining of COL I protein in AVF venous limb, day 1. Scale bar, 40 μ m. **D)** Representative photomicrograph of immunohistochemistry staining of COL I protein in sham vein, day 1. Scale bar, 40 μ m.

A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF

Figure 6.

Collagen III (COL III) expression during AVF maturation. **A)** Bar graph showing mRNA transcripts of COL III in the AVF venous limb relative to sham vein (normalized to day 0; all samples normalized to GAPDH), days 0–42. $n=8$. $P = <.0001$. **B)** Bar graph showing relative quantification of positive immunostaining of COL III protein in the AVF venous limb (normalized to sham vein), day 1. $n=3$. $P = .01$. **C)** Representative photomicrograph of immunohistochemistry staining of COL III protein in AVF venous limb, day 1. Scale bar, 40 μ m. **D)** Representative photomicrograph of immunohistochemistry staining of COL III protein in sham vein, day 1. Scale bar, 40 μ m.

A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF

Figure 7.

Osteopontin (OPN) expression during AVF maturation. **A)** Bar graph showing mRNA transcripts of OPN in the AVF venous limb relative to sham vein (normalized to day 0; all samples normalized to GAPDH), days 0–42. $n=8$. $P < .0001$. **B)** Bar graph showing relative quantification of positive immunostaining of OPN protein in the AVF venous limb (normalized to sham vein), day 1. $n=3$. $P = .02$. **C)** Representative photomicrograph of immunohistochemistry staining of OPN protein in AVF venous limb, day 1. Scale bar, 40 μ m. **D)** Representative photomicrograph of immunohistochemistry staining of OPN protein in sham vein, day 1. Scale bar, 40 μ m.

A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF

Figure 8.

Fibronectin (Fn) expression during AVF maturation. **A)** Bar graph showing mRNA transcripts of Fn in the AVF venous limb relative to sham vein (normalized to day 0; all samples normalized to GAPDH), days 0–42. $n=8$. $P < .0001$. **B)** Bar graph showing relative quantification of positive immunostaining of Fn protein in the AVF venous limb (normalized to sham vein), day 1. $n=3$. $P = .004$. **C)** Representative photomicrograph of immunohistochemistry staining of Fn protein in AVF venous limb, day 1. Scale bar, 40 μ m. **D)** Representative photomicrograph of immunohistochemistry staining of Fn protein in sham vein, day 1. Scale bar, 40 μ m.

A, Artery; V, Vein (non-fistula); FA, Arterial limb of AVF; FV, Venous limb of AVF

Figure 9.

Cartoon depicting the phases of the ECM changes during the adaptive process of AVF maturation. There is an early phase of ECM breakdown from MMP degradation. A transition phase follows with collagens and elastin reorganization the venous scaffolding. Finally, rebuilding of the matrix of the venous limb of the AVF with larger non-collagenous and glycoproteins. TIMP1 and OPN are highly expressed throughout AVF maturation suggesting regulatory roles of these proteins.

Table 1

Primer sequences

GENE	FORWARD	REVERSE
Collagen I	CAGGCTGGTGTGATGGGATT	AAACCTCTCTCGCCTCTTGC
Collagen II	CCAAGACCTGAAACTCTGCCA	GACGGTCTTGCCCCACTTAC
Collagen III	AAATGGGTCCAGCTGGCATT	TCCAGCTACTCCTCTGGGTC
Collagen IV	TGCCTGGAATGATTGGTCCC	CCCATCATGCCTGGACAAGT
Collagen VIII	TGCCAAGAATGGGCAAGGAA	TTTCCCGATGCCAGGAAGTC
Collagen XVIII	GGAGACCCCTACCTGAGAAG	GTCCCGGAAGAAGAGTTTGG
MMP-2	CCTTGACCAGAACACCATCG	TTTAAAGGCCCGAGCAAAAG
MMP-3	TGTGCTCATCTACCCATTGC	CAGGCCCATCAAAAGGGACA
MMP-9	GAAGCGGACATTGTCATCCA	ACATGGGGCACCATTGAGT
MMP-12	AACTTGCAGTCGGAGGGAAC	GAGTTGTCCAGTTGCCAGT
TIMP1	TCATGGGTTCCCCAGAAATC	ACTGCGGTTCTGGGACTTGT
TIMP2	CACGCTTAGCATCACCCAGA	GTCAGCCTTCTTACGGGTCC
TIMP3	CAAGGGCCTCAATTACCGCT	GTTAGTGTCTGGGACGCTCG
TIMP4	GCAAAGACCCTGCTGACACT	GAGACACTCATTGGGGGCTG
Elastin	GTGGTATTGGTGGCATCGGT	CTGGAGCCTTGGCTTTGACT
Fibrillin-1	CCGGATATAACAGGGACCGC	GGTGGTTACCCTCACACTCG
Fibronectin	GACCTGGGAAAAGCCCTACC	CATTGCCTGTGCAGACACAC
Laminin (alpha-2)	GCTCATTTTCAGCTGGGTGGA	CAGATTCGGCACTGAGGGTT
Osteopontin	CCTCCCTCCCGGTGAAAGTG	AAGCTATCACCTCGGCCGTT
Perlecan	ACTCTCAGCTAGAGACCCG	GTAGGTGTCCCCGACTTTG
Syndecan-2	AGCCACATCCCTGGGGAATA	CAGCAATGACGGCTGCTAGA
Thrombospondin-2	ATGGTGAACCAGCTGAGCAA	TGCAGGTGAGCAGGTGATCT
Versican	GGTGGCCCAGAACGGAATA	CAGAGTGTACCTGCTGGTGG
Vitronectin	GATGTCTGGGGCATTGAGGG	CACCTTCCCTGGCACACCAT

Table 2

Gene expression in the AVF compared to sham veins

Top 20 genes with increased expression		
Gene	Description	Fold-change
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch	23.0065
Myh4	myosin, heavy polypeptide 4, skeletal muscle	18.5023
Ckm	creatine kinase, muscle	18.3619
Cthrc1	collagen triple helix repeat containing 1	17.2859
Pvalb	parvalbumin	16.8971
Tnnt3	troponin T3, skeletal, fast	15.4737
Spp1	secreted phosphoprotein 1	14.3271
Acta1	actin, alpha 1, skeletal muscle	13.908
H19	H19 fetal liver mRNA	13.5072
Col12a1	collagen, type XII, alpha 1	13.1818
Ccl8	chemokine (C-C motif) ligand 8	11.4931
Actn3	actinin alpha 3	11.0726
Neb	nebulin	10.6953
Thbs2	thrombospondin 2	9.89351
P4ha3	procollagen-proline, 2-oxoglutarate 4-dioxygenase	9.35871
Casq1	calsequestrin 1	8.18983
Ttn	titin	8.02537
Col8a1	collagen, type VIII, alpha 1	7.87065
Cd109	CD109 antigen	7.75203
Timp1	tissue inhibitor of metalloproteinase 1	7.66475
Top 20 genes with decreased expression		
Phospho1	phosphatase, orphan 1	-5.25613
Inmt	indolethylamine N-methyltransferase	-5.52924
Ppara	peroxisome proliferator activated receptor alpha	-5.59055
Otop1	otopetrin 1	-5.82338
Cpt1b	carnitine palmitoyltransferase 1b, muscle	-5.94216
Nat8l	N-acetyltransferase 8-like	-6.00026
Acacb	acetyl-Coenzyme A carboxylase beta	-6.01774
Pank1	pantothenate kinase 1	-6.06223
Paqr9	progesterin and adipoQ receptor family member IX	-6.21457
Gpd2	glycerol phosphate dehydrogenase 2, mitochondrial	-6.3037
Acot11	acyl-CoA thioesterase 11	-6.33374
Hk2	hexokinase 2	-6.39975
Slc2a5	solute carrier family 2 (facilitated glucose transporter)	-6.64221
Aspg	asparaginase homolog (S. cerevisiae)	-6.68798

Top 20 genes with increased expression		
Gene	Description	Fold-change
Elovl6	ELOVL family member 6, elongation of long chain fatty acids	-6.71688
Slc27a2	solute carrier family 27 (fatty acid transporter)	-6.73115
MLXip1	MLX interacting protein-like	-7.08364
Gys2	glycogen synthase 2	-7.34221
Mpz12	myelin protein zero-like 2	-7.61353
Elovl3	elongation of very long chain fatty acids	-14.3221

Table 3

Pathway enrichment analysis – significantly regulated pathways

Pathway	P value
Tricarmonic acid cycle	1.261E-14
Propionate metabolism	3.062E-14
Butanoate metabolism	2.569E-12
Leucine, isoleucine and valine metabolism	7.251E-12
Propionate metabolism	4.774E-10
Regulation of lipid metabolism_Insulin regulation of fatty acid metabolism	1.096E-08
Mitochondrial long chain fatty acid beta-oxidation	1.451E-08
Oxidative phosphorylation	2.705E-08
Mitochondrial unsaturated fatty acid beta-oxidation	2.902E-08
Cell cycle_Chromosome condensation in prometaphase	8.089E-08
Peroxisomal branched chain fatty acid oxidation	3.413E-07
Cell cycle_The metaphase checkpoint	1.018E-06
Ubiquinone metabolism	2.895E-06
Lysine metabolism	3.211E-06
Glycogen metabolism	3.735E-06
Cell cycle_Role of APC in cell cycle regulation	5.013E-06
Regulation of lipid metabolism_PPAR regulation of lipid metabolism	9.517E-06
Tryptophan metabolism	1.145E-05
Mitochondrial ketone bodies biosynthesis and metabolism	2.869E-05
Lysine metabolism	3.796E-05
Tryptophan metabolism	4.485E-05
Regulation of metabolism_Role of Adiponectin in regulation of metabolism	2.262E-04
CoA biosynthesis	3.457E-04
Cell adhesion_ECM remodeling	3.915E-04
Development_Leptin signaling via PI3K-dependent pathway	4.232E-04
Pyruvate metabolism/ Rodent version	4.340E-04
Mechanism of action of DGAT1 inhibitors in obesity and diabetes mellitus	6.559E-04
Triacylglycerol biosynthesis in obesity and diabetes mellitus, type II	6.559E-04
Putative pathways for stimulation of fat cell differentiation by Bisphenol A	8.159E-04
Cell cycle_Spindle assembly and chromosome separation	1.049E-03
Pyruvate metabolism	1.103E-03
Pentose phosphate pathway	1.112E-03
Saturated fatty acid biosynthesis	1.120E-03
Regulation of lipid metabolism_Insulin regulation of glycogen metabolism	1.532E-03
Regulation of lipid metabolism_Insulin signaling:generic cascades	1.920E-03
Prostaglandin 2 biosynthesis and metabolism	2.104E-03
Pentose phosphate pathway	2.704E-03

Pathway	P value
Cell cycle_Initiation of mitosis	2.817E-03

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Table 4

ECM pathway genes with increased expression

Gene	Description	Fold-increase in AVF compared to sham
TIMP1	Metalloproteinase inhibitor 1	7.66475
MMP-12	Macrophage metalloelastase	6.85608
MMP-14	Matrix metalloproteinase-14	5.56155
PAI1	Plasminogen activator inhibitor 1	4.94775
COL I	Collagen I	4.69459
Versican	Versican core protein	4.38695
COL III	Collagen alpha-1(III) chain	4.11747
SERPINE2	Glia-derived nexin	3.6539
Fibronectin	Fibronectin	3.27387
MMP-2	72 kDa type IV collagenase	3.21068
PLAT (TPA)	Tissue-type plasminogen activator	2.51051
Stromelysin-1	Stromelysin-1	2.3037
CD44	CD44 antigen	2.12833
COL IV	Collagen IV	2.00994

Table 5

ECM component grouping

Proteases	Protease Inhibitors	
- MMP-2	- TIMP1	
- MMP-3	- TIMP2	
- MMP-9	- TIMP3	
- MMP-12	- TIMP4	
Collagens (COL)	Glycoproteins/Non-collagenous proteins	
- COL I	- Elastin	- Perlecan
- COL II	- Fibrillin-1	- Syndecan-2
- COL III	- Fibronectin	-
- COL IV	Thrombospondin-2	
- COL VIII	- Laminin- α 2	- Versican
- COL XVIII	- Osteopontin	- Vitronectin

Table 6

ECM Genes RT-PCR Results

GENE	Day 1	Day 3	Day 7	Day 21	Day 42	Maximal expression*
PROTEASES						
MMP-2			↑			15
MMP-3	↑					188
MMP-9	↑					5
MMP-12			↑			100
PROTEASE INHIBITORS						
TIMP-1	↑	↑	↑	↑		15
TIMP-2				↑		7
TIMP-3				↑		6
TIMP-4				↑	↑	2.5
COLLAGENS						
Collagen I			↑			15
Collagen III		↑	↑			16
Collagen IV			↑	↑		4
Collagen VIII			↑			27
Collagen XVIII			↑			4
GLYCOPROTEINS & NON-COLLAGENOUS PROTEINS						
Elastin		↑	↑			22
Fibrillin-1		↑	↑			8
Fibronectin				↑		12
Laminin (alpha-2)						-
Osteopontin	↑	↑	↑	↑	↑	41
Perlecan				↑		4
Syndecan-2				↑		4.5
Thrombospondin-2			↑			20
Vitronectin				↑		5
Versican		↑		↑		9.5
<div>↑</div> <div></div> <div></div> <div></div>						- Significant increase (p < .05) - At least 2.5-fold increase, not statistically significant - No change - Decrease from baseline, not statistically significant

* maximal fold increase of AVF venous limb compared to sham vein