

SPECIFIC AIMS

Hemodialysis remains a chronic and lifesaving treatment for hundreds of thousands of patients with end stage renal disease (ESRD) in the US. An autogenous dialysis access (arteriovenous fistula, AVF) is the gold standard for delivery of adequate hemodialysis and has been shown to reduce morbidity and mortality compared to grafts (AVG) and catheters. The use of AVFs has increased, yet a significant number fail, either through primary failure to mature or shortly after initiation of dialysis (early failure) and most AVFs develop stenoses in the venous limb necessitating invasive procedures to keep the access functional.

The creation of an AVF directly connects two vascular beds that are physiologically fundamentally differently equipped to accommodate various types of flow and pressures, and thereby forces adaptation upon both the arterial and venous segments of the fistula. This process ideally leads to a mature fistula that is able to sustain hemodialysis. However, maladaptation of the access vein is frequent given the change in flow and pressure patterns, the uremic milieu, the pro-inflammatory state of dialysis patients and the surgical injury during access creation. The most common cause for access dysfunction is neointimal hyperplasia (NIH) in the venous segment of the access leading to stenosis and ultimately thrombosis. The current treatment for stenoses in an autogenous access is angioplasty. This is an effective treatment in the short term but re-stenosis is almost universal and repeat procedures are necessary in nearly all cases thereby increasing costs of access maintenance as well as exposing patients to procedural dangers.

There is a limited understanding of how flow induced gene expression patterns cause maladaptive changes in the venous wall culminating in vascular stenosis. In endothelial cells exposed to disturbed flow, histone deacetylases (HDACs) are key regulators of oxidative, inflammatory, and proliferative responses, in part through Krüppel-like factor 2 (KLF2), an important transcription factor that has anti-inflammatory and atheroprotective activity. We hypothesize that HDACs and KLF2 in endothelial cells are central to regulating venous adaptation to flow and maladaptation causes hyperplasia and stenosis.

A better understanding of the molecular events connecting flow and hyperplasia will identify new targets for medical treatment to improve fistula patency and lead to better dialysis with improved survival.

Specific issues that will be addressed

Disturbed flow pattern is a major contributor to maladaptive vascular remodeling and causes molecular changes leading to local inflammation, proliferation, migration and matrix deposition.

We will map areas that are exposed to specific flow patterns (disturbed flow with low and oscillating wall shear stress [WSS] versus pulsatile flow) for the expression of histone deacetylases (HDACs) and the atheroprotective transcription factor Krüppel-like factor 2 (KLF2) and correlate this with proliferative activity and development of neointimal hyperplasia. We will use endothelial specific knockout mice (HDAC KO driven by tie2 promoter in vascular endothelial cells) and HDAC inhibitors to further delineate the role of HDACs in venous stenosis.

Specific Aim #1: To determine the expression patterns of HDACs 1/2/3 and 5/7 and KLF2 in venous segments subjected to laminar flow and segments with high shear stress (pulsatile flow upstream after fistula), and correlate expression patterns to proliferation, cell type and neointimal hyperplasia.

Specific Aim #2: To determine the causative effect of HDAC on venous vascular wall shear stress induced changes by blockage using HDAC inhibitors and specific knockout in vascular endothelial cells.

Hypothesis: HDACs and KLF2 are differentially expressed in areas of laminar flow versus segments with high shear stress. High shear stress in the vein is associated with HDAC activation and KLF2 down-regulation and subsequently causes proliferation, cell migration and intimal hyperplasia. These effects can be mitigated by HDAC blockage.

Experimental model and proposed approach

High shear stress in veins will be induced using the established aorto-caval fistula model. Flow patterns before tissue harvesting will be determined by ultra-high frequency doppler ultrasound and harvested tissue from regions exposed to different flow patterns will be subjected to immunofluorescence- and immunochemistry-staining for HDACs and KLF2 as well as expression mapping by real-time quantitative reverse transcriptase PCR. Staining for cellular proliferation, inflammatory cell markers, smooth muscle cell markers and collagen deposition is used to determine vessel wall changes.

SIGNIFICANCE AND INNOVATION

Vascular access is the *sine qua non* to facilitate hemodialysis in patients that have reached end stage renal disease (ESRD). AVFs are the gold standard and preferred access for patients who undergo hemodialysis and have been shown to be associated with the lowest morbidity and mortality of all vascular access types (1). However, up to 60% of AVFs fail to mature, as a consequence of maladaptive response to increased flows, intra-access pressures and shear stress leading up to occlusive neointimal hyperplasia (NIH) (2). The current proposal will establish a molecular signature of maladaptive changes with focus on HDACs and KLF2 in veins exposed to high shear stress and correlate these with the development of intimal hyperplasia and stenosis. The molecular events in the venous system that is exposed to high shear stress are poorly understood and the proposed work will describe a basic molecular regulatory framework based on knowledge from the arterial system. Understanding of regulatory states associated with development of intimal hyperplasia and correlation with shear stress will identify pharmacological targets for intervention. We anticipate that these data will eventually identify viable treatment approaches that reduce restenosis and improve AVF patency and access lifetime. This will benefit patient health and quality of life, and will lead to a decrease in number and frequency of invasive procedures as well as a reduction in overall costs of care.

Histone deacetylases (HDACs) have been shown to regulate molecular processes in response to blood flow conditions in endothelial cells. HDACs are a class of enzymes that remove acetyl groups from ϵ -N-acetyl lysine amino acid on a histone and thereby regulate gene expression in a number of target genes. Histone deacetylation by HDACs will result in tightly wrapped DNA and transcriptional repression. HDACs also target many non-histone proteins and, in certain cases, directly cause gene activation. Important groups of genes regulated in the vasculature include genes implicated in pro-inflammatory signaling pathways and genes that regulate proliferation and migration. HDACs are ideal targets for intervention because a number of specific inhibitors have been developed that are used to regulate HDAC activity and a large number of genes involved in common deleterious pathways can be differentially targeted by an intervention, e.g. inhibition of a specific HDAC or a class of HDACs.

Krüppel-like factor-2 (KLF2) is a critical mediator of protection from shear stress in endothelial cells. Pulsatile shear stress induces KLF2 mediating the enhancement of vessel compliance and confers antithrombotic, anti-adhesive, and anti-inflammatory effects. The expression pattern of KLF2 in the venous system under different flow conditions is unknown but given the importance of KLF2 as a molecular switch for regulating gene expression and proliferation in other tissues it is highly likely that KLF2 plays a major role in adaptation of veins to high flow conditions.

While both HDACs as well as KLF2 expression are known to be important in the genesis of atherosclerosis, their role in the venous system is not studied. We hypothesize an essential role for HDACs and KLF2 in venous flow adaptation and maladaptation and postulate that they are prime targets for intervention on a pharmacological basis.

Innovative new treatments to prevent venous flow maladaptation can be designed based on detailed knowledge about molecular events leading to intimal hyperplasia and stenosis. HDACs and KLF-2 are ideal targets given the large amount of genes regulated by them and the availability of clinically relevant inhibitors.

Practical considerations:

AVFs are the gold standard for dialysis access but patency is frequently threatened by stenosis, which is recurrent in most cases and eventually leads to loss of access. The molecular events in maladaptation of the venous system to high flow and development of stenosis are unknown for the most part. Based on data from the arterial system, HDACs and KLF2 are promising candidates for intervention on a molecular level. HDAC inhibitors are already being used clinically making HDACs a practical target for future directed therapy approaches.

RESEARCH STRATEGY

Overview

Arteriovenous fistulas (AVFs) are the preferred mode of vascular access in dialysis patients and are associated with the lowest rate of complications such as infections and thrombosis. AVFs have decreased morbidity and mortality rates compared to grafts and catheters and this translates into improvement in quality of life for patients.

The major problem associated with the creation of an AVF is the failure to mature secondary to NIH. NIH develops based on a molecular cascade of events that start with a change in flow pattern. An increase in flow in the venous segment, for which the vein is not physiologically equipped, as well as a disturbed flow pattern at the arterial side expose endothelial cells, mainly in the segment of the anastomosis, to an altered flow pattern which triggers molecular signaling directed towards rectifying the situation. These events can be either adaptive or maladaptive. Adaptive events cause the vessel to grow outward and maintain the lumen diameter but equipping it for the changed flow pattern. Maladaptive events, on the other side, cause an inward growth with narrowing lumen diameter causing an ever-changing flow pattern ultimately leading to NIH. Upstream and downstream events leading to the process of NIH generation have been identified (3). Upstream events are triggered by the initial endothelial injury (surgery) and shear stress as well as needle punctures and include platelet activation/adhesion, leukocyte recruitment and smooth muscle cell (SMC) migration/proliferation. The endothelium is taking a lead role in this process and coordinates events by sending molecular signals. Downstream events are mainly SMC migration and proliferation but also inflammatory reactions triggered by leukocyte recruitment.

The specific locations of stenotic lesions are evidence that disturbed flow patterns are triggers for reactive events that lead to NIH. Endothelial cells are at the intersection between blood flow and the cellular compartment of the vessel wall and are thereby in a privileged position to coordinate and direct signals about the intravascular state towards the supportive structures of the vessel wall.

Laminar shear stress in arteries triggers a protective molecular cascade of events in endothelial cells starting with activation of calmodulin dependent kinases, which cause HDAC5 phosphorylation, as well as expression of Krüppel-like factor 2 (KLF2). KLF2 triggers enhanced eNOS (endothelial nitric oxide synthase) expression, which has a protective effect on arteries. While this cascade protects cells exposed to laminar shear stress, the response to disturbed flow patterns is different and resembles the response to atherogenesis including leukocyte adhesion/transmigration, increased expression of adhesion molecules and reactive oxygen species (ROS) all triggering pro inflammatory pathways leading to cell proliferation and a maladaptive response pattern.

More commonly NIH develops at the venous side of the anastomosis. This is in part because venous endothelial cells are adapted to a low flow pattern and respond to disturbed flow with a pattern that resembles the response to atherogenesis.

KLF2 and HDAC5 and HDAC7 expression have been linked to protection from damage in a pulsatile flow situation through decreased proliferation of cells as well as induction of eNOS, whereas other members of HDACs are linked to maladaptive events such as increased cell proliferation and decreased eNOS expression (4). KLFs are a subclass of the zinc finger family of DNA-binding transcription factors and KLF2 expression is limited to endothelial cells within the vessel wall. Endothelial cell specific knock out of KLF2 causes high-output heart failure through profound loss of vascular tone but the mechanism is not entirely known. KLF2 acts as a key "molecular switch" regulating important aspects of vascular function and disease. Intriguingly, KLF2 expression is decreased at vascular branch points which are the earliest regions of the human vasculature shown to develop atherosclerotic lesions. KLF2 inhibits NF- κ B function at multiple levels and thereby causes a general anti-inflammatory response. An overview of key functions of KLF2 is given in table 1 (modified from (5)).

Table 1: KLF2 targets

Key KLF2 targets	Function
<i>Decrease</i> VCAM-1, E-selectin	Anti-inflammatory
<i>Increase</i> eNOS	
<i>Decrease</i> PAI-1, Tissue Factor	Anti-thrombotic
<i>Increase</i> TM, eNOS, tissue Plasminogen Activator	
<i>Decrease</i> Endothelin-1	Vasodilatory
<i>Increase</i> C natriuretic peptide, eNOS, Arginino succinase synthase	
<i>Decrease</i> VEGF-R2	Anti-angiogenic
<i>Increase</i> semaphorin-3F	

HDACs promote chromatin condensation and transcriptional repression but also target non-histone proteins and can participate directly in gene activation. HDACs are major factors governing epigenetic regulation of gene expression, which has been recognized to be important in AVF maturation (6). HDACs 1/2/3 have been linked to a maladaptive response with disturbed/oscillatory flow causing CyclinA increase and p21 decrease resulting in increased cell proliferation and decrease in KLF2 expression. Other functions and binding partners of HDACs involved in regulation of flow response are listed in table 2.

Table 2: Overview of HDACs

	Substrates/targets	Binding partners	Tissue expression	Knockout
HDAC1	P53, MyoD, E2F-1, Stat3, androgen,	Sin3, Mi-2/NuRD, CoREST	ubiquitous	Embryonic lethal
HDAC2	Bcl-6, Stat3, glucocorticoid receptor, YY-1	Sin3, Mi-2/NuRD, CoREST	ubiquitous	Fatal cardiac defects
HDAC3	GATA-1, RelA, Stat3, MEF2D, YY-1, SHP	N-CoR/SMRT	ubiquitous	Embryonic lethal
HDAC5	Smad7, GCMa	HP-1, REA, estrogen receptor	Heart, smooth muscle, brain	Viable, abnormal cardiac stress response
HDAC7	FLAG1 and 2	HIF1a, Bcl-6	Heart, placenta, smooth muscle	Embryonic lethal

In summary, pulsatile flow elicits a protective response in endothelial cells with KLF2 and HDAC5 and 7 being major factors mediating this response whereas oscillatory (disturbed) flow causes a maladaptive response leading to atherosclerosis and possibly NIH with HDAC 1/2/3 involvement. We hypothesize that these factors play a major role in AVF maladaptation to high flow conditions and that inhibiting certain HDACs and augmenting KLF2 response will lead to a novel therapeutic approach towards improved AVF maturation and patency.

Experimental Design

Specific Aim #1: To determine the expression patterns of HDACs 1/2/3 and 5/7 and KLF2 in venous segments subjected to laminar flow and segments with high shear stress (pulsatile flow upstream after fistula), and correlate expression patterns to proliferation, cell type and neointimal hyperplasia.

Hypothesis: HDACs and KLF2 are differentially expressed in areas of laminar flow versus segments with high shear stress. High shear stress in the vein is associated with HDAC activation and KLF2 down-regulation and subsequently causes proliferation, cell migration and intimal hyperplasia. These effects can be mitigated by HDAC blockage.

Rationale

Intimal hyperplasia and subsequent stenosis is the major cause of failure in AVFs. Intimal hyperplasia develops preferentially at specific sites that are subjected to a turbulent or oscillatory flow pattern. Laminar shear stress (LSS) (pulsatile flow) causes expression of vasoprotective factors such as HDAC5 and 7 and KLF-2 in endothelial cells. While this differential expression pattern is well described in arteries and has been shown to contribute to atherogenesis (4), there are no data in AVFs. Flow patterns change in all segments after AVF creation and this also changes expression patterns of adaptive factors. Given the importance of HDACs 1/2/3 and 5/7 and KLF2 for adaptive flow responses in arteries we hypothesize that they play an important role regulating flow responses in the venous circuit that is exposed to high shear stress.

The aortocaval mouse model (7) is ideally suited to study the question of molecular changes because it is easy to establish, has a low mortality and fistula size can be varied by using different needle gauges. A recent publication described the changes in this mouse model as recapitulating human fistula development (7).

Strategy

We will use the established aortocaval mouse fistula model to study changes in the venous system upstream of a fistula (7). This model is ideally suited to delineate flow-mediated molecular changes as it

exposes the vein to pulsatile flow and high shear stress, conditions that are characteristically predominant in AVFs. Following the creation of an aortocaval fistula in a mouse, vascular tissue will be harvested at specific time points (early=1, 3, 7 days - late = 4 weeks) from specified sites: venous site subjected to high flow and venous control site with regular flow. Flow patterns (turbulent versus laminar flow) before harvest will be recorded using a flow probe prior to associate expression of HDACs and KLF-2 with specific flow patterns. From each specific site, tissue for frozen sections, paraffin sections as well as tissue for RNA will be obtained and expression patterns of HDACs 1/2/3 and HDAC5 and 7 and KLF2 will be measured by immunofluorescence staining and RT-PCR. Proliferation of endothelial as well as smooth muscle cells and collagen deposition will be correlated to HDAC/KLF2 expression by simultaneous staining for KI-67, Sirius red as well as BrDU (injected 60 minutes before tissue harvest).

Table 3: Schematic overview research plan described in Specific Aim #1:

Time points	Segments	Data collected
Early (1, 3, 7 days after aortocaval fistula creation)	Venous site exposed to high flow	<ul style="list-style-type: none"> Flow pattern (pulsatile versus oscillatory) HDAC expression KLF2 expression
	Venous control site	<ul style="list-style-type: none"> Proliferative index (Ki67, BrDU) Collagen deposition (Sirius Red)
Late (4 weeks after aortocaval fistula creation)		

Expected results and alternative approaches

HDACs and KLF2 have been described as important factors governing the response to flow patterns in the vasculature. We expect that they are expressed differentially before and after fistula creation and that they follow a pattern associated with the change in flow. Furthermore, we expect that proliferation of endothelial and smooth muscle cells is associated with expression of HDACs 1/2/3 and KLF2 expression is associated with a decrease in proliferation and decrease in inflammatory response. The experiments are designed and timed to show the differential expression patterns and allow associations between expression patterns, flow pattern and proliferation to be drawn. The model is established and shown to recapitulate the adaptation to flow similar after human AVF creation (7) Time points are carefully chosen to allow for adaptations to occur. In case the shear stress is not enough to induce the expected changes, a small band will be tied around the IVC to induce a mild stenosis in the high flow area that will mimic a stenosis and elicit turbulent and reciprocal flow conditions. Antibodies are well characterized in the vasculature and staining techniques are established. Alternative approaches will include blotting techniques and expression mapping by molecular methods.

Specific Aim #2: To determine the causative effect of HDAC on venous vascular wall shear stress induced changes by blockage using HDAC inhibitors and specific knockout in vascular endothelial cells.

Hypothesis: HDACs and KLF2 are differentially expressed in areas of laminar flow versus segments with high shear stress. High shear stress in the vein is associated with HDAC activation and KLF2 down-regulation and subsequently causes proliferation, cell migration and intimal hyperplasia. These effects can be mitigated by HDAC blockage.

Rationale

Exposure of the venous system to high and reciprocal shear stress causes molecular changes that are intended to be adaptive to the increased stress on the vessel wall but regularly turn maladaptive by causing intimal hyperplasia and subsequent stenosis threatening the patency of the fistula. Specific aim 1 is designed to delineate molecular changes with special emphasis on factors regulating gene expression. In order to establish a causative relationship between expression of HDACs and maladaptive changes including intimal hyperplasia, collagen deposition and proliferation of smooth muscle cells, blocking studies are necessary to show cause and effect. HDAC inhibitors are used in cancer treatment in clinical trials and are safe and effective. HDAC inhibitors interfere with HDAC activity and regulate cell cycle, differentiation and apoptosis in cancer cells. HDAC inhibitors also not only target histones yet have the ability to influence a number of processes including cell cycle arrest, angiogenesis, immune modulation and

apoptosis by targeting non-histone proteins. While this is an advantage over therapies targeted at a single pathway it also has the potential to cause more unwanted side effects.

Further studies using knockout mice with endothelial cell specific knockdown of single HDACs are ideally suited to determine the effect of an individual HDAC on such a complicated process as intimal hyperplasia yet are beyond the time and scope of this proposal but are mentioned as a next logical step.

Strategy

In order to determine the effect of HDAC inhibition on fistula maturation and intimal hyperplasia, administration of HDAC inhibitors are used in the aortocaval fistula model at the time of fistula generation. We focus on four different inhibitors that are specific for certain Class I or Class II HDACs. These are listed in table 4. HDAC inhibitors are injected intra-peritoneally at the time of surgery and measured outcomes are proliferation, intimal hyperplasia, area of collagen deposition. Experimental and control groups (each at least n=6) include: HDAC inhibitor injection, control substance injection (usually substrate used to dissolve HDAC inhibitor, e.g. DMSO), sham surgery, HDAC injection with sham surgery.

Table 4: HDAC inhibitors used in this study

HDAC Inhibitor	Target (specificity ++)	Demonstrated Clinical Effects
Valproic acid	HDAC1(++), HDAC2(++), CoREST(++), NuRD(++) HDAC3(+), NCoR(+)	induces differentiation of carcinoma cells; tumor growth and metastasis formation are significantly reduced
PCI-334051	HDAC8(++)	induces caspase-dependent apoptosis, induces cytochrome c release from mitochondria
Entinostat	HDAC1, HDAC3 (both are Class I HDACs)	induced apoptosis in B-cells, modulating immunosuppressive cells
TMP269	HDAC 4/5/7/9 (Class IIa HDACs)	inducing apoptosis in cancer cells

Expected results and alternative approaches

Based on literature review we expect HDAC inhibition to decrease intimal hyperplasia formation after fistula generation and reduce proliferative activity of smooth muscle cells as well as inhibit collagen deposition thereby improving adaptation to high flow conditions in the venous system after fistula generation. HDAC inhibitors have been shown to have beneficial effects in cancer treatment by inducing apoptosis, inhibiting cell cycle progression and synergistic effects with cytotoxic agents. Although there are no data available in the venous system, different groups of HDACs have been described in regulating the oxidative, inflammatory, and proliferative responses to disturbed flow of endothelial cells in the arterial system. An advantage of factors modifying gene expression is that not only one pathway is affected and targets are multifaceted thereby having a broader angle and a higher chance to be effective. This on the other side can also be a disadvantage as complex systems are harder to analyze and cause and effect are not always clear from the experimental design. We have designed the experiments carefully as to maximize the knowledge gained and minimize potential overlaps. The experimental mouse model is established and recapitulates the human adaptation to fistula development including failure of fistula development. The mouse model is chosen because of the availability of transgenic strains that will allow for tissue-specific knockout of HDACs in future experiments to delineate further the effects of single HDACs that are beyond the scope of this proposal. There are alternative models but they are either technically difficult to perform with a high failure rate and higher costs and provide no clear advantage over the model chosen in this proposal. For the case that a simple increase in flow after aortocaval fistula creation does not induce the expected changes we will implement a banding of the inferior vena cava proximal to the fistula in order to induce an external stenosis mimicking disturbed flow conditions including reciprocal flow.

Timeline

Given the chosen timelines of experimental follow up with late groups followed 4 weeks we expect each experimental design to be completed in about 3 months, which includes tissue processing, staining, data collection and analysis. Specific aim 1 is reasonably completed within 6 months leaving another 6

months for specific aim 2. Animal group sizes (at least 10 animals per experimental group) are chosen large enough to account for unexpected and expected deaths (perioperative mortality about 10%) so that there will be at least 6 animals per group for statistical analysis.

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FACILITIES AND RESOURCES

Personnel

PI: Florian Toegel, MD, PhD
Nephrology Research Fellow, BWH

Key personnel : Dirk Hentschel, MD
Director of Interventional Nephrology, BWH

Equipment and laboratory research space

The PI has access to research space located in the Harvard Institute of Medicine (HIM) on the 5th floor with shared resources as delineated below.

LABORATORY. The laboratory (~3,000 square feet) is located in the Harvard Institutes of Medicine, a state-of-the-art research facility shared by Brigham and Women's Hospital, Beth Israel Deaconess Hospital, and the Harvard Institute of Human Genetics. The 10-story, 300,000 squarefoot facility includes both basic science and clinical divisions of the Harvard Medical School and affiliated institutions and is fully equipped for studies in cell biology, molecular biology, biochemistry, and biophysics. All of the equipment for the studies proposed is available in the laboratory, the laboratories of close collaborators, and/or local core facilities affiliated with Brigham and Women's Hospital and/or the Harvard Stem Cell Institute. There is sufficient bench space and desk space within the lab for the proposed project. Available general equipment includes autoclave, centrifuges, 4, -20, and -80 degree freezers, liquid nitrogen storage space, fluorescence and light microscopy, real-time PCR, as well as the necessary small equipment (gel boxes, power supplies, incubators, etc). To prepare tissues for histology a cryostat is available and paraffin sections will be done by the pathology service. Bench space, imaging facilities including a confocal imaging microscope and a epifluorescence microscope as well as a light microscope all equipped with cameras for image capture and software are available on HIM 5th floor.

ANIMALS. Within the Brigham and Women's Hospital complex, 33,338 square feet of space are devoted to the housing of various species used in biomedical research. Cages of mice bred and used by the lab are housed in a full barrier, limited BL2-N facility on the basement floor of the Harvard Institutes of Medicine. A surgical area fully equipped for small animal surgery including a surgical microscope, heating pads with temperature monitoring equipment, lights and an operating area is located in the HIM 5th floor as a common resource.

OFFICE. The office area consists of a dedicated office space and ~150 sq. ft. of Renal Division shared office space used for photocopying, facsimile transmission, and storage of office supplies.

COMPUTER. Computational resources for preparation of manuscripts, figures and scientific correspondence are currently present in the laboratory. Two HP Laserjet printers are usable by all laboratory members.

Blood flow studies

Studies to measure flow patterns will be done using a VisualSonics Vevo 2100 ultrasound that is available at the Small Animal Imaging Core Laboratory (Children's Hospital, Director: Treves, S. Ted, M.D.). The use of this high frequency ultrasound system has been validated in mice by one of our collaborators, Dr Keith Ozaki (Reference: Murine ultrasound imaging for circumferential strain analyses in the angiotensin II abdominal aortic aneurysm model. Favreau JT, Nguyen BT, Gao I, Yu P, Tao M, Schneiderman J, Gaudette GR, Ozaki CK. J Vasc Surg. 2012 Aug;56(2):462-9).