

Flow-Dependent Remodeling of Small Arteries in Mice Deficient for Tissue-Type Transglutaminase

Possible Compensation by Macrophage-Derived Factor XIII

Erik N.T.P. Bakker, Adrian Pistea, Jos A.E. Spaan, Titia Rolf, Carlie J. de Vries, Nico van Rooijen, Eleonara Candi, Ed VanBavel

Abstract—Chronic changes in blood flow induce an adaptation of vascular caliber. Thus, arteries show inward remodeling after a reduction in blood flow. We hypothesized that this remodeling depends on the crosslinking enzyme tissue-type transglutaminase (tTG). Flow-dependent remodeling was studied in wild-type (WT) and tTG-null mice using a surgically imposed change in blood flow in small mesenteric arteries. WT mice showed inward remodeling after 2 days of low blood flow, which was absent in arteries from tTG-null mice. Yet, after continued low blood flow for 7 days, inward remodeling was similar in arteries from WT and tTG-null mice. Studying the alternative pathways of remodeling, we identified a relatively high expression of the plasma transglutaminase factor XIII in arteries of WT and tTG-null mice. In addition, vessels from both WT and tTG-null mice showed the presence of transglutaminase-specific crosslinks. An accumulation of adventitial monocytes/macrophages was found in vessels exposed to low blood flow in tTG-null mice. Because monocytes/macrophages may represent a source of factor XIII, tTG-null mice were treated with liposome-encapsulated clodronate. Elimination of monocytes/macrophages with liposome-encapsulated clodronate reduced both the expression of factor XIII and inward remodeling in tTG-null mice. In conclusion, tTG plays an important role in the inward remodeling of small arteries associated with decreased blood flow. Adventitial monocytes/macrophages are a source of factor XIII in tTG-null mice and contribute to an alternative, delayed mechanism of inward remodeling when tTG is absent. (*Circ Res.* 2006;99:86-92.)

Key Words: vascular remodeling ■ transglutaminase ■ resistance arteries ■ macrophages ■ blood flow

Hemodynamic parameters such as blood flow and blood pressure are considered important stimuli in the adaptation of arterial caliber.¹⁻⁴ In small arteries, low blood flow and high blood pressure are both associated with a reduction in lumen diameter.⁵⁻⁸ A structural reduction in diameter necessarily involves a reorganization of both cellular and extracellular matrix components around a smaller lumen. The mechanisms of such arterial remodeling are poorly understood. Yet, a reduction in lumen diameter dramatically decreases maximal perfusion attributable to their fourth-power relationship and clinically, inward remodeling is associated with an increased risk of cardiovascular events.⁹

Transglutaminases are Ca²⁺-dependent enzymes that induce protein crosslinking via ϵ -(γ -glutamyl)lysine bonds. There are at least 8 family members, of which tissue-type transglutaminase (tTG) (Tgase 2) is ubiquitously expressed.¹⁰ Consequently, the biological actions of tTG are studied most intensely. These studies revealed its dual role as a crosslinking enzyme and G protein. The biological functions of tTG

involve extracellular matrix organization, protein crosslinking, α_1 -adrenergic signaling, and activation of RhoA through transamidation.¹¹⁻¹³ The crosslinks that are formed by transglutaminases are highly resistant to mechanical challenge and proteolytic degradation. These properties suggest that tTG could play multiple roles in vascular structure and function.

Using primarily an in vitro approach, we recently found evidence for the important role of transglutaminase activity in matrix reorganization and inward remodeling of small arteries.¹⁴ This work generated the hypothesis that these enzymes are also involved in the in vivo remodeling in response to hemodynamic stimuli and that specifically tTG is involved. In the present study, we therefore addressed this hypothesis using a genetic model, the tTG-null mouse. These mice are viable and display a mild phenotype, which includes impaired wound healing.¹⁵ Mice deficient for tTG show normal values for heart rate, cardiac contractility, and blood pressure.¹⁶ However, vascular properties and, in particular, the response to a vascular remodeling stimulus have not been studied in

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From the Departments of Medical Physics (E.N.T.P.B., A.P., J.A.E.S., T.R., E.V.) and Medical Biochemistry (C.J.d.V.), Cardiovascular Research Institute Amsterdam, Academic Medical Center; Department of Molecular Cell Biology (N.v.R.), Faculty of Medicine, Vrije Universiteit, Amsterdam, the Netherlands; and Biochemistry Laboratory (E.C.), IDI-IRCCS, c/o Department of Experimental Medicine, University of Rome Tor Vergata, Italy.

Correspondence to Erik N. T. P. Bakker, Department of Medical Physics, Academic Medical Center and Cardiovascular Research Institute Amsterdam, PO Box 22700; 1100 DE Amsterdam, the Netherlands. E-mail n.t.bakker@amc.uva.nl

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these mice. Here, we aimed to elucidate the role of tTG in the remodeling of resistance arteries in response to increased or decreased blood flow.

Materials and Methods

Mice

Mice deficient for tTG and wild-type (WT) littermates were obtained from G. Melino (University of Rome Tor Vergata, Italy). Mice are from a mixed Svj129/C57Bl/6 background and were bred at the local animal facility. PCR analysis confirmed genotype of tTG-null and WT animals. Mice were fed ad libitum and had free access to drinking water. Male and female mice, equally distributed among groups, were used for experiments at the age of 3 to 5 months. All experiments were approved by the local committee for animal experiments.

To determine contractile responses, mesenteric arteries of untreated mice were mounted on stainless steel wires in a Mulvany–Halpern myograph (Danish Myo Technology). The bath contained physiological saline solution (PSS) that was bubbled with 95% air/5% CO₂ and kept at 37°C. Vessels were normalized according to standard procedures⁵ and activated twice with PSS containing 120 mmol/L K⁺ (KPSS) for 5 minutes. A concentration–response relationship was recorded for phenylephrine (3×10⁻⁸ to 3×10⁻⁵ mol/L). The concentration of phenylephrine was increased stepwise every 5 minutes. Responses were normalized to the maximal response obtained with KPSS.

Flow-Induced Remodeling

Mesenteric arteries, first- or second-order branches from the superior mesenteric artery were subjected to a surgical procedure to modify blood flow (supplemental Figure I, available online at <http://circres.ahajournals.org>), which is a modification of the procedure used previously in rats.⁸ Mice were anesthetized with a mixture of ketamine (100 mg/kg), xylazine (10 mg/kg), and atropine (0.05 mg/kg) injected IP. After an incision through the skin and abdominal muscles, a loop of the bowel was exteriorized. One single artery was ligated in the middle and at the distal end with 7-0 sutures. The segment in between the ligations was excised and placed in calcium-free MOPS buffer. This yielded the normal-flow segment (NF). The bowel was placed back into the abdomen and the wound was stitched in 2 layers, closing muscles and skin, using 7-0 sutures. A subcutaneous injection with Temgesic (2 mg/kg) was given to reduce pain. After 2 or 7 days of altered blood flow, the mice were euthanized. The mesentery was then excised and placed in cold calcium-free MOPS buffer. The ligated segment exposed to low blood flow (LF) and the adjacent high blood flow (HF) artery were then dissected. The isolated vessels were mounted in a pressure myograph as described previously,¹⁴ and a pressure-diameter relationship was determined in the presence of papaverine (0.1 mmol/L). For reasons of comparison, additional experiments were done according to the flow modifying procedure used previously in rats,¹⁴ which involves single ligation of one artery. This low-flow artery was compared with the adjacent artery exposed to high blood flow and a control artery exposed to normal blood flow (supplemental Figure I).

Monocyte/Macrophage Depletion

The role of peritoneal macrophages was studied in tTG-null mice during a 7-day period of reduced blood flow. Mice were given 3 consecutive injections with 0.2 mL liposome-encapsulated clodronate to deplete the peritoneal cavity from macrophages.¹⁷ Injections were given 3 days before surgery, immediately after surgery, and 3 days after surgery. Liposome-encapsulated clodronate was prepared as previously described.¹⁸ Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany). Monocytes/macrophages were identified using Giemsa staining and quantified by averaging counts from 2 blinded observers.

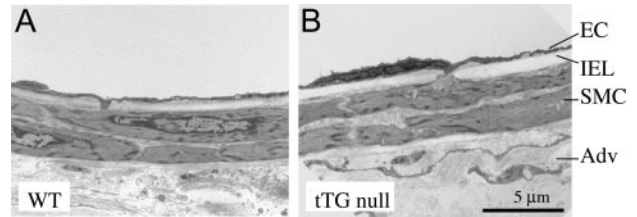


Figure 1. Electron microscopic images of mesenteric arteries from WT (A) and tTG-null (B) mice. Arteries were isolated, cannulated, and fixed at 80 mm Hg. No qualitative differences were observed between arteries from WT and tTG. EC indicates endothelial cell; IEL, internal elastic lamina; SMC, smooth muscle cell; Adv, adventitia.

Immunohistochemistry

Arteries were frozen in Tissue-tek and sectioned. After overnight drying, sections were fixed in acetone for 5 minutes. Sections were blocked with 10% normal goat serum (Dako Cytomation) for 15 minutes and then incubated for 60 minutes with an antibody against tTG (rabbit polyclonal antibody Ab-4 [Neomarkers]; concentration 1:20) or an antibody against the ε-(γ-glutamyl)lysine crosslink [mouse monoclonal antibody 811-MAG [Covablab]; concentration 1:160]. The secondary antibodies used were a polyclonal goat anti-rabbit antibody (Dako Cytomation; concentration 1:100) and a goat anti-mouse IgG1 antibody (Southern Biotech; concentration 1:100), both applied for 30 minutes. Sections were quantified by a blinded observer on a scale from 0 (absent) to 3 (strong). Negative controls were obtained in the absence of the primary antibody.

Reverse-Transcription PCR

Mesenteric small arteries from WT and tTG-null mice were dissected in MOPS buffer at 4°C. Blood was removed from the vessel lumen. Arteries (length ≈15 mm) were immersed in 1 mL of TRIzol (Gibco) for RNA extraction. cDNAs were subsequently synthesized using an Omniscript reverse transcriptase kit (Qiagen). Real-time PCR was performed in iQ SYBR Green Supermix buffer (Bio-Rad) using a MyCycler (Bio-Rad) thermal cycler. Primers for mouse transglutaminases 1 to 6, factor XIIIa, and RPLP0 gene (housekeeping gene) were designed using Beacon Designer software (PREMIER Biosoft) and purchased from Invitrogen. PCR cycles were: 15 seconds at 95°C (denaturation), 15 seconds at 60°C (annealing), and 30 seconds at 72°C (elongation) for a total of 40 cycles, with fluorescence recording at the end of each elongation step. Primer details are listed in supplemental Table I.

Statistical Analysis and Calculations

Data are expressed as mean±SEM. Data were compared by paired or unpaired Student's *t* test and Bonferroni correction where appropriate. Differences were considered significant at *P*<0.05. Circumferential strain was calculated as $(D - D_0)/D_0$, where *D* is the observed lumen diameter for a given intraluminal pressure and *D*₀ is the original diameter measured at 3 mm Hg. Circumferential stress was calculated as $(P \cdot D)/(2W)$, where *P* is intraluminal pressure, and *D* and *W* are lumen diameter and wall thickness, respectively.

Results

Mice, Vessel Function, and Morphology

WT mice (n=26) were slightly bigger than tTG-null mice (n=34): 33±1 g versus 30±1 g (*P*<0.05). Untreated mesenteric arteries from WT mice and tTG-null mice were similar in size: 220±7 μm versus 216±7 μm (at 80 mm Hg). Electron microscopic images of mesenteric arteries showed no qualitative differences (Figure 1). Thus, both vessels from WT and tTG-null mice showed a continuous layer of endothelial cells, with occasional connections to smooth muscle

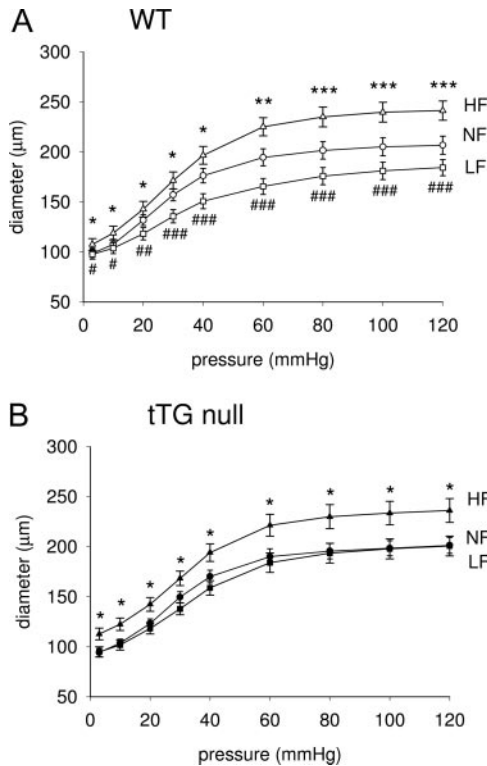


Figure 2. Passive pressure–diameter relationship of mesenteric arteries 2 days after surgically imposed changes in blood flow in WT mice (A) and tTG-null mice (B). Arteries from tTG-null mice lack inward remodeling as observed in WT mice. Vessel segments exposed to normal flow (NF) were excised on day 0. Low-flow (LF) segments were obtained from the same artery on day 2. Adjacent arteries were exposed to high flow for 2 days (HF). $N=6$ to 8 vessels per group. $*P<0.05$, $**P<0.01$, $***P<0.001$ for NF vs HF; $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ for NF vs LF.

cells. The media consisted of a 2-cell-thick layer of smooth muscle cells, delineated by an internal elastic lamina and a discontinuous external elastic lamina. The adventitia mainly consisted of collagen fibers, fibroblasts, and occasional monocytes/macrophages. Contractile properties of mesenteric arteries were tested in a Mulvany–Halpern myograph. Responses to the α_1 -adrenergic agonist phenylephrine were similar in arteries of WT and tTG-null mice (supplemental Figure II).

Flow-Induced Remodeling

Mice subjected to a blood flow–modifying intervention recovered well from surgery. Wound healing appeared to be similar in WT and tTG-null mice. The mice demonstrated differences in vascular remodeling in response to decreased blood flow (Figure 2). In WT mice, after 2 days of continuous exposure to low blood flow (LF), vessels had remodeled inwardly in comparison to normal flow (NF). When measured after isolation and cannulation, a reduction in passive lumen diameter was found over the whole pressure range (3 to 120 mm Hg). For instance, at 80 mm Hg a diameter reduction of $17\pm 3\%$ was found ($P<0.01$). In contrast, in tTG-null mice inward remodeling in response to LF was absent. There was no difference in lumen diameter over the whole pressure

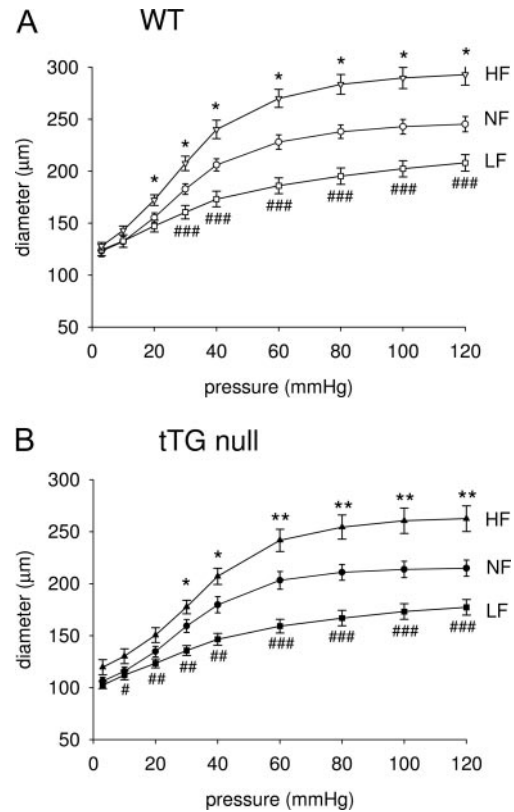


Figure 3. Passive pressure–diameter relationship of mesenteric arteries 7 days after surgically imposed changes in blood flow in WT mice (A) and tTG-null mice (B). In contrast to the results at day 2, arteries from tTG-null mice now show inward remodeling to low blood flow. Vessel segments exposed to normal flow (NF) were excised on day 0. Low-flow (LF) segments were obtained from the same artery on day 7. Adjacent arteries were exposed to high flow for 7 days (HF). $N=6$ vessels per group. $*P<0.05$, $**P<0.01$, $***P<0.001$ for NF vs HF; $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ for NF vs LF.

range (3 to 120 mm Hg). For instance, at 80 mm Hg, a difference of $0\pm 2\%$ was found ($P<0.001$ for WT versus tTG). The vessels exposed to high blood flow (HF) showed similar outward remodeling in WT and tTG-null mice. Thus, at 80 mm Hg an increase in lumen diameter of $17\pm 2\%$ and $19\pm 5\%$ was found for WT and tTG-null mice, respectively ($P=NS$).

After a prolonged exposure to altered blood flow of 7 days, differences between WT and tTG-null mice had disappeared (Figure 3). Vessels from tTG-null mice now demonstrated similar inward remodeling to low flow as compared with WT mice: $18\pm 1\%$ versus $21\pm 2\%$ reduction in lumen diameter at 80 mm Hg for WT and tTG-null, respectively ($P=NS$). Outward remodeling to high flow was similar: $20\pm 5\%$ and $21\pm 3\%$ for WT mice and tTG-null mice, respectively ($P=NS$).

Stress–strain relationships were constructed for vessels from WT and tTG-null mice after 2 days or 7 days of altered blood flow. In WT mice, low flow was associated with reduced distensibility (ie, higher stress for equal strain), whereas high flow was accompanied by an increased distensibility. Such changes in the stress–strain

Wall-to-Lumen Ratio and Wall Cross-Sectional Area of Mesenteric Arteries Subjected to Altered Blood Flow

	Normal Flow	2 Days		7 Days	
		LF	HF	LF	HF
Wall/lumen					
WT	0.099±0.002	0.160±0.008***	0.119±0.008	0.182±0.010***	0.099±0.005
KO	0.118±0.004	0.152±0.003***	0.124±0.008	0.222±0.026***	0.120±0.004
wCSA					
WT (%)	100	112±6	179±21**	125±11	140±16*
KO (%)	100	133±4**	151±17*	123±11	149±12**

Arteries were analyzed after low blood flow (LF) or high blood flow (HF) for 2 days and 7 days. The wall cross sectional area (wCSA) was normalized to the normal flow vessel. Statistical significance is indicated by **P*<0.05, ***P*<0.01, and ****P*<0.001 tested against normal flow.

relationship were not observed in tTG-null mice after 2 days. After 7 days, a decrease in distensibility was found with low flow in both WT and tTG. An increase in distensibility was found in WT vessels exposed to high flow, and only a tendency to an increased distensibility was found in vessels from tTG-null mice exposed to high flow (supplemental Figure III).

In both WT and tTG-null mice a highly significant increase in wall-to-lumen ratio was found in response to low blood flow, both after 2 days and 7 days (Table). In WT mice, the increase was mainly attributable to the reduction in diameter. The increase in wall-to-lumen ratio after 2 days of low blood flow in tTG-null mice, however, was the result of an increase in wall cross sectional area, not of a reduced lumen diameter. Additional morphometric analysis on cross sections indicated that the media cross-sectional area was not significantly increased: 107±11% as compared with normal flow (n=5). However, a large number of adventitial monocytes/macrophages could be observed (see also Figure 6). Although in vessels exposed to high blood flow, the lumen diameter increased in all groups, the wall-to-lumen ratio was maintained in all cases. Thus, the increase in lumen diameter associated with HF in both WT and tTG-null mice was accompanied by an increase in wall cross sectional area.

Compensatory Mechanisms in tTG-Null Mice

The data presented above demonstrate that inward remodeling is delayed in the tTG-null mouse and that, at least in the case of low blood flow, alternative mechanisms of remodeling are available. Immunohistochemistry indicated positive staining for tTG protein in mesenteric arteries of WT mice. Particularly intense staining was seen in the endothelium and adventitia, whereas more diffuse staining was observed in the media (Figure 4). Arteries from tTG-null mice stained negatively for tTG protein. However, vessels from both WT and tTG-null mice stained positively for the ε-(γ-glutamyl)lysine crosslink. Similar to tTG protein in WT mice, particularly the endothelium and adventitia stained positively for the crosslink, whereas a more modest staining of the media was observed. These findings suggest that transglutaminase activity is still present in tissues from tTG-null mice, and,

therefore, alternative mechanisms of vascular remodeling may involve other transglutaminase family members. As current information on the expression of transglutaminases in vascular tissue is limited, we used RT-PCR to quantify mRNA levels of all known transglutaminases. Whereas transglutaminases 3 to 6 were below detection limit, both WT and tTG-null mice showed the expression of type 1 transglutaminase and a relatively high expression of the plasma

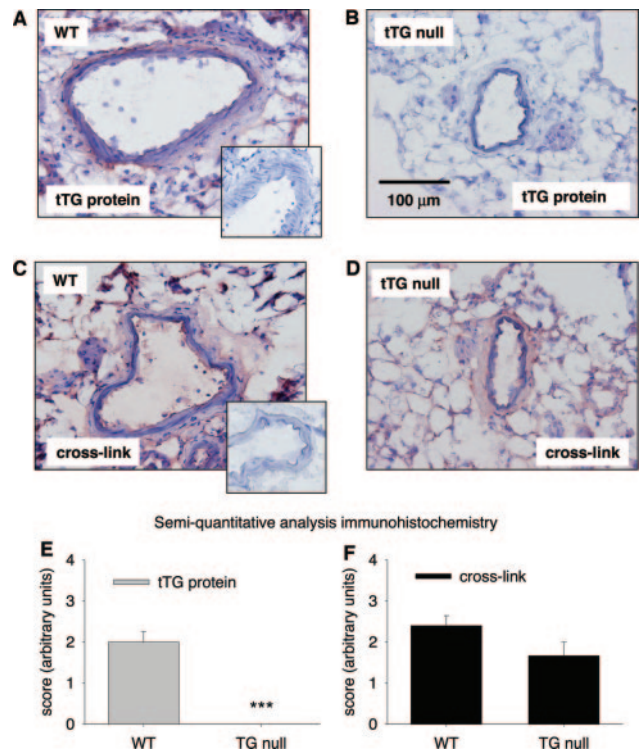


Figure 4. Immunostaining of mesenteric arteries from WT (A) and tTG-null (B) mice for tTG protein and the ε-(γ-glutamyl)lysine bond (C and D). Staining for tTG antigen (brown) in WT mice is particularly strong in the endothelium and adventitia and intermediate in the media. In vessels from tTG-null mice staining for tTG protein is negative. Vessels from both WT and tTG-null mice stain positively (brown) for the ε-(γ-glutamyl)lysine bond. N=3 to 6 per group. Insets in A and C are negative controls. Quantification of immunostaining for tTG protein and crosslink is shown in E and F. ****P*<0.001 for WT vs tTG null.

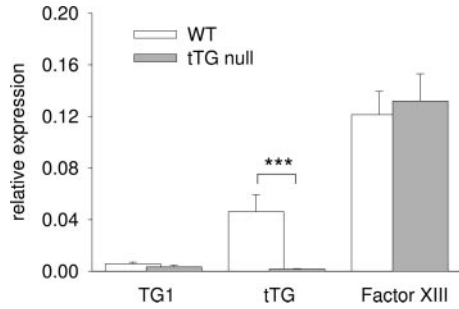


Figure 5. Expression of transglutaminases in mesenteric arteries from WT and tTG-null mice. RT-PCR revealed the expression of type 1, tTG, and plasma transglutaminase (factor XIII). Types 3, 4, 5, and 6 transglutaminase were not detectable. Arteries from tTG-null mice do not show differences in the expression of transglutaminases other than tTG. N=6 to 9 vessels per group. *** $P < 0.001$ for WT vs tTG null.

transglutaminase factor XIII (Figure 5). None of the transglutaminases were upregulated in tTG-null mice as compared with WT mice.

Based on the prominent expression of factor XIII and the presence of the ϵ -(γ -glutamyl)lysine crosslinks in small mesenteric arteries, we next focused on this plasma transglutaminase as a potential backup system in tTG-null mice. Because only monocytes/macrophages are known to express factor XIII in vascular tissue, tTG-null mice were treated with liposome-encapsulated clodronate to deplete the peritoneum from macrophages. We found that monocytes/macrophages are normally present in the perivascular region and accumulate in the adventitia of low-flow vessels in the tTG-null mouse (Figure 6). Treatment with liposome-encapsulated clodronate reduced the expression

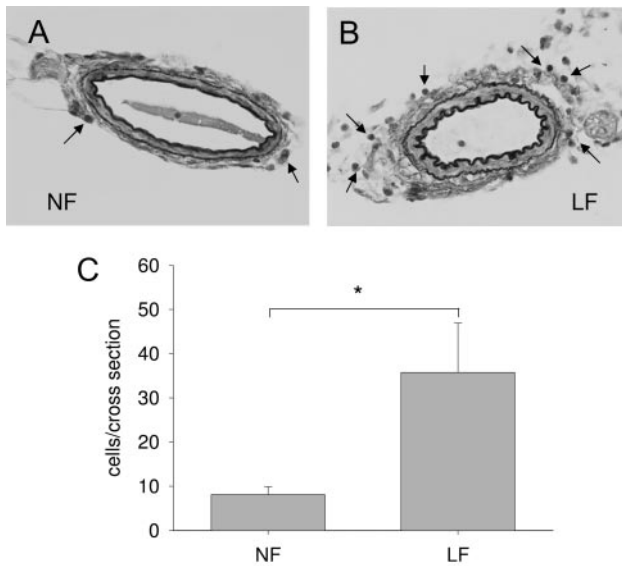


Figure 6. Monocytes/macrophages are present in the adventitia of control vessels from tTG-null mice (A) and accumulate in vessels exposed to low blood flow (B). A significant increase in the number of monocytes/macrophages per cross-section (C) was found around low-flow vessels (LF; n=9) as compared with normal flow vessels (NF) (n=8). * $P < 0.05$.

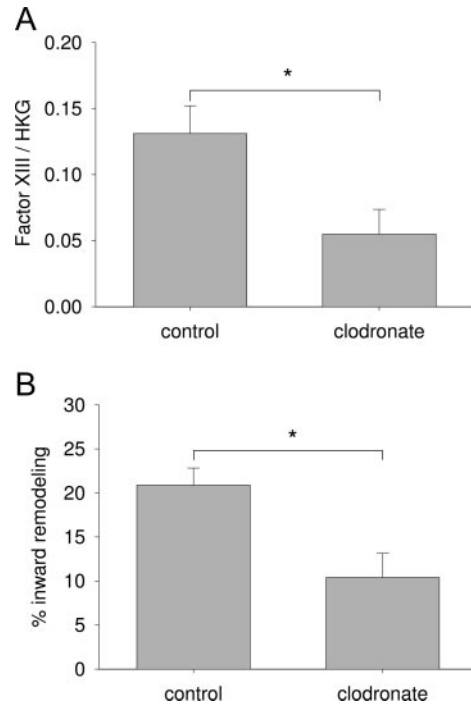


Figure 7. The expression of factor XIII in mesenteric arteries is decreased after treatment of mice with liposome-encapsulated clodronate (A). Concomitantly, inward remodeling is reduced after 7 days of low blood flow in tTG-null mice after treatment (B). N=6 to 9 in each group. * $P < 0.05$ for control vs treatment with liposome-encapsulated clodronate.

of factor XIII and concomitantly reduced inward remodeling in the tTG-null mice after 7 days of low blood flow (Figure 7).

Discussion

This study demonstrates that tTG is involved in small artery inward remodeling after flow impairment. Because the tTG-null mice developed normally, and because inward remodeling following flow reduction was delayed but not suppressed in these mice, backup mechanisms must be present. We additionally demonstrate here that adventitial monocytes/macrophages account for the redundant mechanisms of inward remodeling, most likely through the release of the plasma transglutaminase factor XIII.

Initial evidence for a role of transglutaminases in inward remodeling came from our recent study,¹⁴ which used primarily in vitro approaches. Thus, in that study, we observed that small coronary arteries and skeletal muscle arteries showed inward remodeling in vitro on exposure to exogenous transglutaminase. Manipulation of endogenous transglutaminase activity and expression modulated the remodeling of small arteries kept in organoid culture. That initial work on transglutaminases in remodeling, together with the current demonstration of the role of specifically tTG using the knock-out model and factor XIII as a backup mechanism, underline the importance of specific members of this enzyme family in a variety of conditions and species.

Arterial remodeling in response to a change in blood flow is essential for adequate tissue perfusion. Evidence suggests that the remodeling to an increase in blood flow is triggered by a change in shear stress¹⁹ and the subsequent release of endothelium-derived factors such as nitric oxide.²⁰ Indeed, our work on isolated small coronary arteries in organoid culture confirm this notion.²¹ Studies in knockout mice revealed that the cytoskeletal proteins dystrophin²² and vimentin²³ participate in remodeling, although tissue-ACE²⁴ also plays a role. The present study connects to these studies and provides a mechanism, based on transglutaminases, which can effectuate the inward remodeling of small arteries in response to reduced blood flow. The model that we used herein has previously been applied to mice by Loufrani et al²² and exploits the arcading network of the mesenteric arteries feeding the intestine. The arcading arteries that were used in the present study are 150 to 300 μm and, therefore, should be regarded as resistance arteries.²⁵ Remodeling was assessed by comparison of the lumen diameter of arteries exposed to normal blood flow and altered blood flow. This comparison relies on the assumption that arteries are initially similar in size. To avoid a possible bias from such biological variation, we developed a strategy to study remodeling within 1 single artery. We, therefore, performed a double ligation in a single artery, excised the segment between both ligations at day 0, and harvested the remaining part after several days of flow reduction. This yields a control and intervention segment from the same artery. With this strategy, we observed a very consistent decrease in lumen diameter with low blood flow. The inward remodeling is similar in the current approach, albeit with much lower variation, as compared with the approach that uses single ligation and matched arteries: $-17 \pm 3\%$ ($n=6$) versus $-12 \pm 10\%$ ($n=4$) at 80 mm Hg for WT arteries exposed to low blood flow for 2 days (supplemental Figure D). Arteries that are under longitudinal strain may not be suitable for this approach, because the results may be complicated by simultaneous longitudinal remodeling.²⁶

We did not address the functional consequences of the current ligation model with respect to myogenic reactivity and agonist-induced responses, as done by others in rats.²⁷ Therefore, additional work is needed at this point. The functional consequence of the lack of tTG was studied by comparison of the contractile responses to the α_1 -agonist phenylephrine. Despite the notion that tTG may act as a G protein in adrenergic signaling,¹¹ we observed no differences in contractile responses.

Compensatory Mechanisms in tTG-Null Mice

Although the current data demonstrate a dominant role for tTG in inward remodeling to reduced blood flow, it is clear from the 7-day experiments that alternative mechanisms are present. We found several lines of evidence indicating that these alternative mechanisms involve other transglutaminase family members. This evidence is based on (1) mRNA expression of Tgases, (2) the presence of the ϵ -(γ -glutamyl)lysine bonds, and (3) the decrease in factor XIII expression and inhibition of inward remodeling after monocyte/macrophage depletion.

Several transglutaminase isoenzymes have been isolated and characterized at the protein level in mammals. These include plasma transglutaminase factor XIII, keratinocyte transglutaminase (type 1), the ubiquitous tTG (tissue type), the epidermal/hair follicle transglutaminase (type 3), the prostatic transglutaminase (type 4), and types 5 to 7 transglutaminase.^{11,28} We found that type 1, tTG, and plasma transglutaminase (factor XIII) are expressed in mesenteric arteries of mice. Type 1, the keratinocyte transglutaminase, has only recently been identified in vascular endothelial cells.²⁹ The identification of factor XIII expression in resistance arteries was unexpected, because neither endothelial cells nor smooth muscle cells nor fibroblasts are known to express factor XIII. Because our data indicate that factor XIII expression is relatively high in mesenteric arteries, we next focused on this enzyme as a potential backup mechanism in tTG-null mice. Monocytes/macrophages are known to express factor XIII, and we, therefore, studied their role using liposome-encapsulated clodronate.¹⁷ By virtue of their phagocytotic nature, macrophages ingest these liposomes and are killed after intracellular release of the drug. Treatment of tTG-null mice caused a reduction in factor XIII expression in small mesenteric arteries. Thus, this finding is consistent with the idea that monocytes/macrophages represent the source of factor XIII. Associated with this, a significant decrease in the inward remodeling to low blood flow was found. Collectively, these data therefore support the hypothesis that alternative mechanisms of remodeling operate in tTG mice that involve adventitial monocytes/macrophages, which may release factor XIII.

Inflammatory processes are associated with neointima formation in atherosclerosis and the pathophysiology of hypertension.³⁰ In addition, the role of monocytes/macrophages in outward remodeling of collateral vessels or arteriogenesis is described in several studies.² To the best of our knowledge, the role of these cells in inward remodeling of resistance arteries to reduced blood flow is novel. Therefore, this study adds to the versatile nature of monocytes/macrophages and their role in vascular remodeling. Because we did not observe monocytes/macrophages adhering to the endothelium or in the media, the source of these cells could be the peritoneum. The peritoneum is naturally rich in monocytes/macrophages, and, thus, the inward remodeling of mesenteric arteries may be different from other vascular beds where the monocytes/macrophages need to be derived directly from the bloodstream. In addition, because we only subjected tTG-null mice to clodronate treatment, future work is needed to address the role of monocytes/macrophages in WT animals and other conditions associated with inward remodeling.

Monocytes/macrophages are known to display quite opposite phenotypes, depending on the specific context.³¹ Thus, "classically" activated macrophages promote tissue breakdown through the release of matrix metalloproteinases and reactive oxygen species, whereas "alternatively" activated cells are profibrotic and are involved in tissue repair. Interestingly, factor XIII expression has been proposed as a marker of alternatively activated macro-

phages.³² The potential to influence vascular caliber to a substantial extent in quite opposite ways depict macrophages as cells of primary interest in vascular biology. Clearly, much more work is needed to unravel the regulation of monocyte/macrophage phenotype in relationship to vascular remodeling.

A particularly intriguing question that remains to be addressed is how changes in blood flow are linked to the activity of transglutaminases. An attractive hypothesis is that altered levels of nitric oxide modulate the activity of transglutaminase. Both tTG and the plasma transglutaminase factor XIII are directly inhibited through nitrosylation of critical cysteine residues.^{33,34} We previously showed that the remodeling induced by exogenous factor XIII is completely blocked in the presence of a nitric oxide donor.¹⁴ Thus, on a decrease in shear stress, the reduced availability of nitric oxide could “unlock” the active site of the enzyme and lead to enhanced transglutaminase activity within the vessel wall. Obviously, additional experiments are needed to further substantiate this hypothesis.

In conclusion, our findings suggest that tTG and factor XIII play crucial roles in the inward remodeling of small arteries. Transglutaminases, acting as biological glue, provide a mechanism to effectuate a structural reduction in vessel diameter. This mechanism may play a role both in normal regulation of vascular caliber and in pathological conditions associated with constrictive arterial remodeling.

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Disclosures

None.

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Flow-Dependent Remodeling of Small Arteries in Mice Deficient for Tissue-Type Transglutaminase: Possible Compensation by Macrophage-Derived Factor XIII
Erik N.T.P. Bakker, Adrian Pisteu, Jos A.E. Spaan, Titia Rolf, Carlie J. de Vries, Nico van Rooijen, Eleonara Candi and Ed VanBavel

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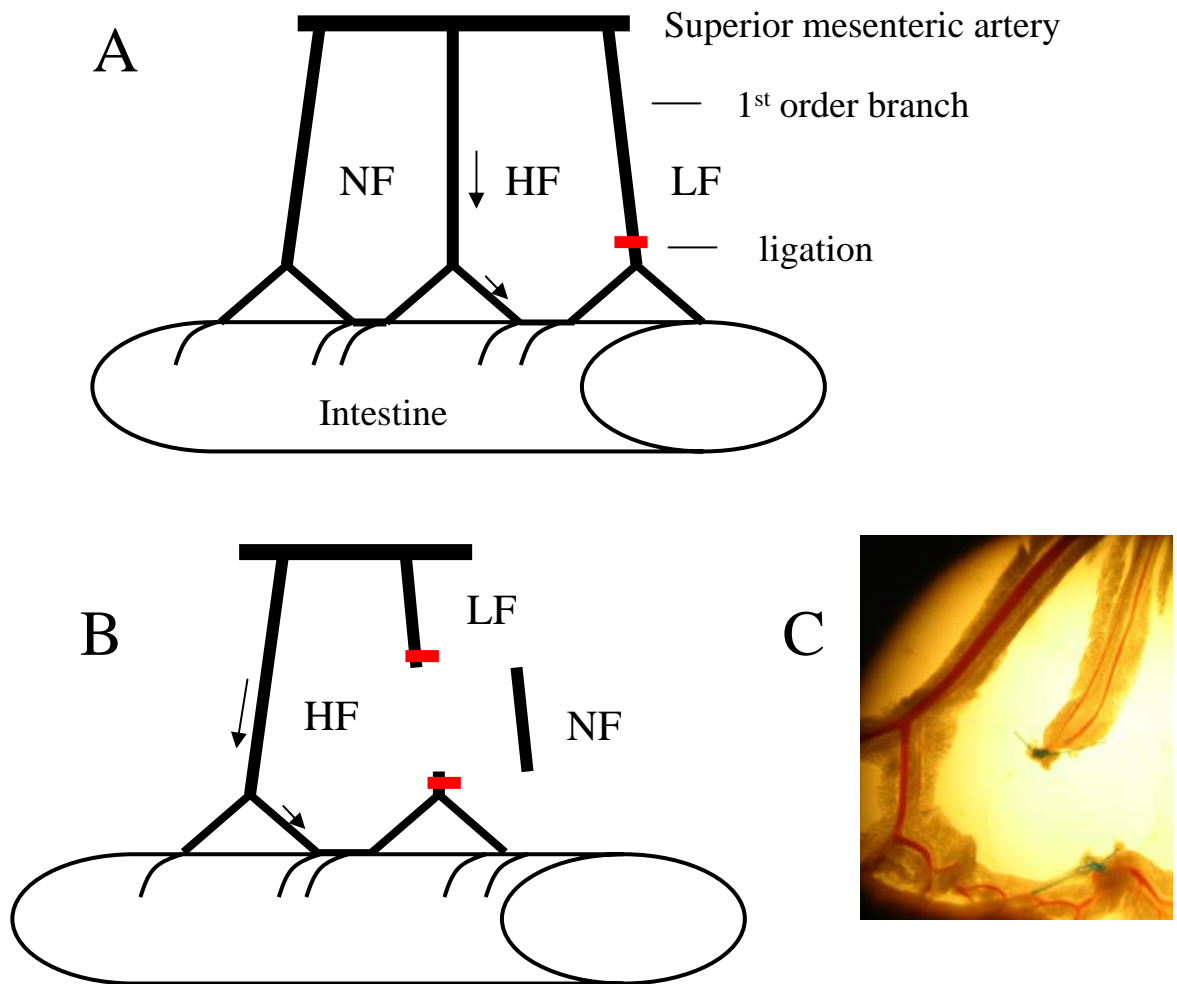
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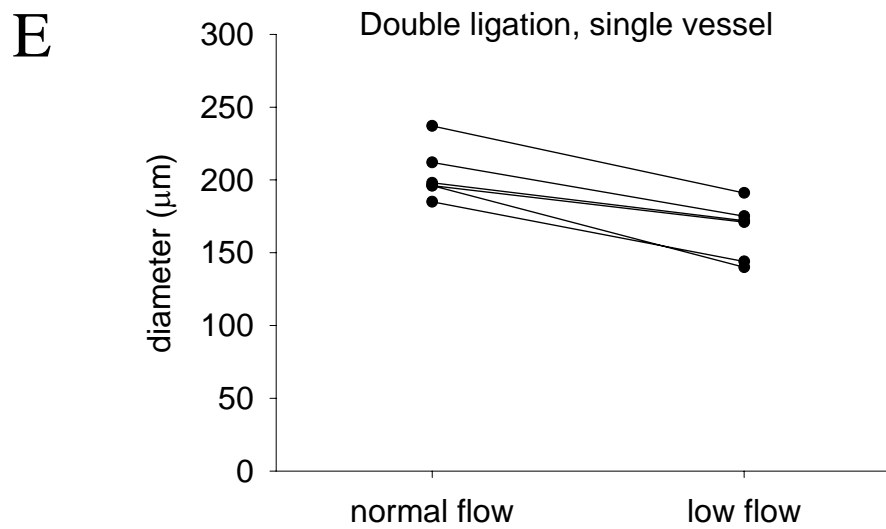
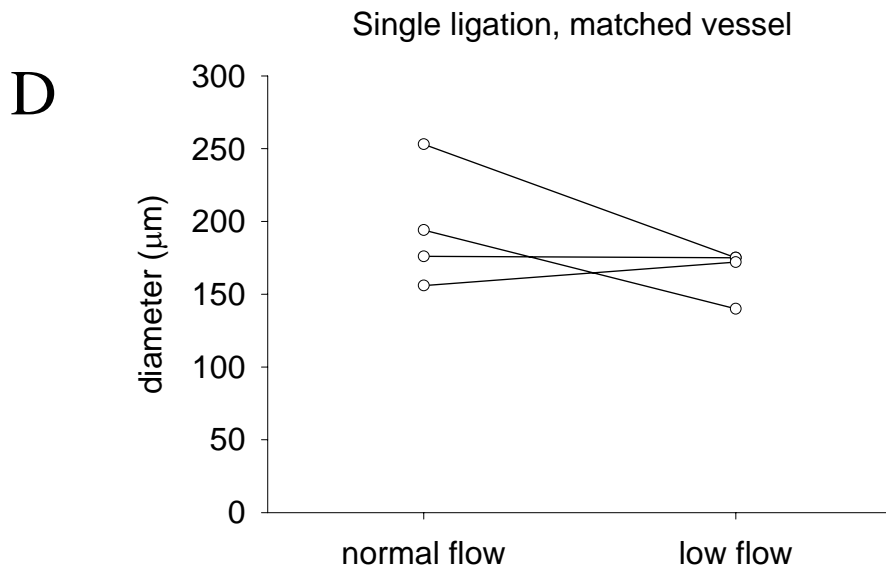
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Online Figure 1. Flow-modifying procedures



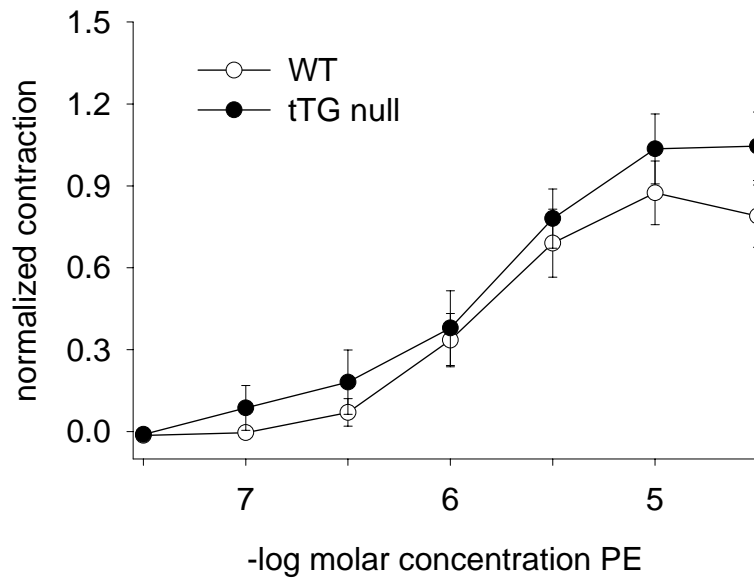
Schematic representation of mouse mesenteric arteries. The conventional procedure (A) to change blood flow uses a single ligation to create a low flow (LF), high flow (HF), and a matched normal flow (NF) vessel. The present study uses a double ligation to create a paired NF and LF vessel from a single artery (B). Thus, the NF segment is excised on day 0 and the remaining LF segment is excised after 2 or 7 days of recovery and compared to the NF segment to determine remodeling. Arrows indicate compensatory blood flow via collaterals, which creates a HF vessel. Panel C shows an image of the actual procedure.

Online Figure 1. Flow-modifying procedures



Comparison of two surgical approaches to reduce blood flow. Both a matched vessel (D) and a single vessel (E) approach show the inward remodeling of low flow vessels. Due to the truly paired comparison, the single-vessel approach shows a very consistent inward remodeling to low blood flow.

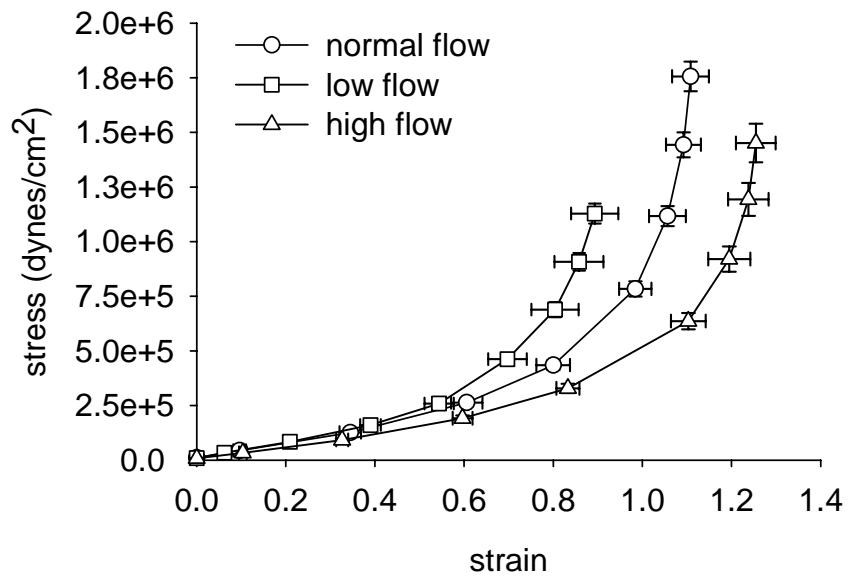
Online Figure 2. Contractile function of mesenteric arteries



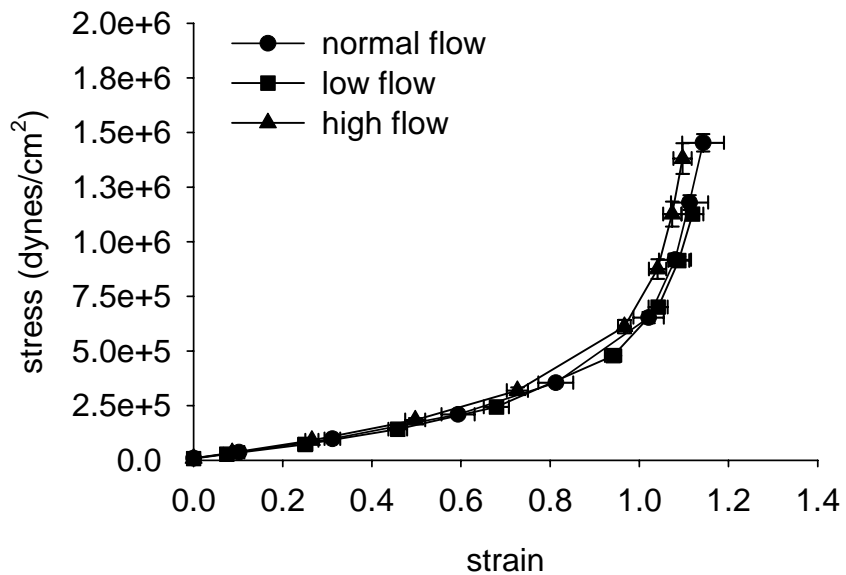
Since tTG may act as a G-protein in α_1 -adrenergic signaling, contractile responses were tested to the α_1 -agonist phenylephrine (PE). Responses were normalized to the maximal response to 120 mM K^+ , which was similar in WT and tTG null mice: 9.1 ± 0.8 mN vs. 7.4 ± 1.5 mN. Contraction to PE was not different in arteries from WT (n=8) and tTG null mice (n=9) when tested in a wire myograph setup.

Online Figure 3. Stress-strain relationships

A: WT 2 days



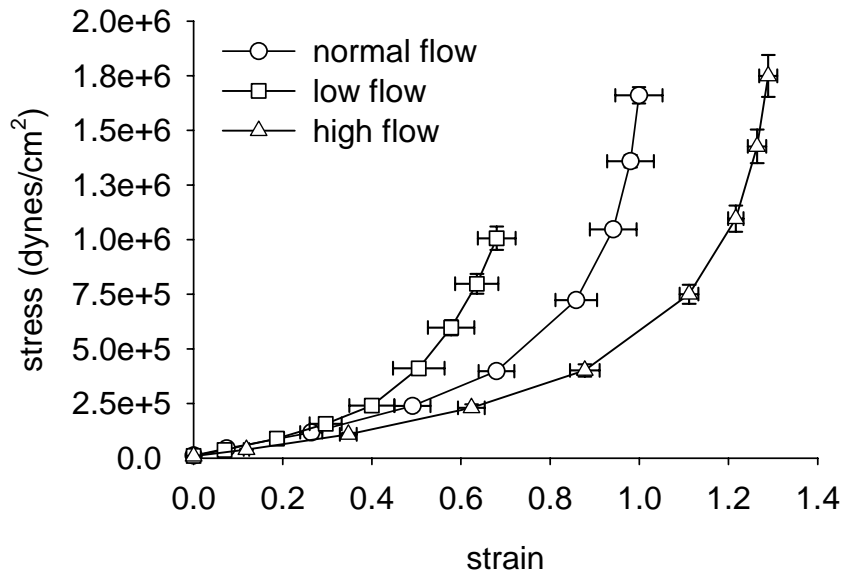
B: tTG null 2 days



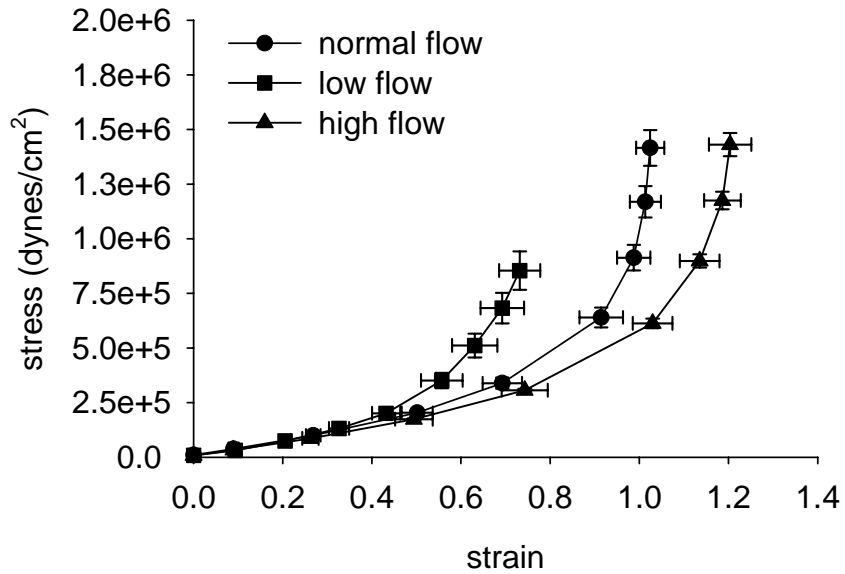
Stress-strain relationships after 2 days of altered blood flow. To compare the curves, stresses were estimated at an interpolated strain of 0.8. In WT mice low flow was associated with increased stress ($p < 0.05$), while high flow was associated with decreased stress ($p < 0.01$) at this level of strain. Such changes were absent in tTG null mice.

Online Figure 3: Stress-strain relationships

C: WT 7 days



D: tTG null 7 days



Stress-strain relationships after 7 days of altered blood flow. In WT mice low flow was associated with increased stress ($p < 0.01$), while high flow was associated with decreased stress ($p < 0.01$) at the same level of strain (0.8). In tTG null mice stress was significantly increased ($p < 0.05$) with low flow, but not significantly changed with high flow at the same level of strain (0.8).

Online Table 1. Nucleotide sequence of primers used for RT PCR together with expected size of amplified products.

Sequence Definition	Product Length	Sense Primer/ Antisense Primer
Mus musculus transglutaminase 1, K polypeptide (Tgm1), mRNA.	114	AGCCTCGGACTCTGTGACC ACCTGCCCACTCTCCTTGAC
Mus musculus transglutaminase 2, C polypeptide (Tgm2), mRNA.	140	GTCCCGTCCAACAATTTCTCG ACAGCACAGCAGCCTCAG
Mus musculus transglutaminase 3, E polypeptide (Tgm3), mRNA.	107	AACTCAAACCTAACGCATCTTTTCG TGCCAGTATCCCCGTGACC
Mus musculus transglutaminase 4 (prostate) (Tgm4), mRNA.	174	GCCTCGCTAACATGAAATCC GCTACTTGCTGATGAGAACG
Mus musculus transglutaminase 5 (Tgm5), mRNA.	197	AGTGATGTCCTCCAGGTCTC AGTGTGATGAATGCTGTGTCC
Mus musculus transglutaminase 6 (Tgm6), mRNA.	146	CACAGTTCCCAGACATCAAGG AGACAGAAGCAGGACCAACC
Mus musculus coagulation factor XIII, A1 subunit (F13a1), mRNA.	156	ACCCTTGAGTGCTGAGAG AACTATAAATCTGCCCTACAAAG
Mus musculus acidic ribosomal phosphoprotein P0, mRNA	85	GGACCCGAGAAGACCTCCTT GCACATCACTCAGAATTTCAATGG