Flow-Dependent Remodeling in the Carotid Artery of Fibroblast Growth Factor-2 Knockout Mice

Chris J. Sullivan, James B. Hoying

Objective—Fibroblast growth factor-2 (FGF2) has been implicated as a mediator in the structural remodeling of arteries. Chronic changes in blood flow are known to cause reorganization of the vessel wall, resulting in permanent changes in artery size (flow-dependent remodeling). Using FGF2 knockout (Fgf2−/−) mice, we tested the hypothesis that FGF2 is required during flow-dependent remodeling of the carotid arteries.

Methods and Results—All branches originating from the left common carotid artery (LCCA), except for the left thyroid artery, were ligated to reduce flow in the LCCA and increase flow in the contralateral right common carotid artery (RCCA). Age- and sex-matched control animals did not undergo ligation of the LCCA branches. Morphometric analysis showed that by day 7, vessel diameter was significantly greater in the high-flow RCCA of FGF2 wild-type (Fgf2+/+) and Fgf2−/− mice versus the respective control RCCA, demonstrating outward remodeling. In contrast, vessel diameter was decreased by day 7 in the low-flow LCCA of both genotypes compared with the control LCCA, showing inward remodeling. No differences were observed between Fgf2+/+ and Fgf2−/− mice in either high-flow or low-flow remodeling.

Conclusions—Given these results, we demonstrate that FGF2 is not essential for flow-dependent remodeling of the carotid arteries. (Arterioscler Thromb Vasc Biol. 2002;22:1100-1105.)

Key Words: arterial remodeling ■ basic fibroblast growth factor ■ knockout mice ■ flow-dependent remodeling ■ fibroblast growth factor-2

Vascular remodeling is the structural reorganization of a vessel involving a variety of cell activities, including proliferation, apoptosis, migration, and extracellular matrix restructuring.1–3 Remodeling of the arterial wall occurs after chronic changes in blood pressure and blood flow and in response to vessel injury.4–7 Arterial remodeling due to changes in blood flow (flow-dependent remodeling) occurs in physiological1,8 and pathological situations.9–12 In pathological settings, such as atherosclerosis and angioplasty, arterial remodeling plays a critical role in the degree of vessel narrowing during plaque or lesion progression.5,13–16

The molecular mediators of vessel remodeling are still unclear. Fibroblast growth factor-2 (FGF2) is a molecule that is strongly implicated in flow-dependent remodeling. FGF2 mRNA expression is sensitive to alterations in fluid flow and shear stress,17 and FGF2 protein expression increases in the vascular wall during flow-induced arterial enlargement.18 In addition, antibody neutralization of endogenous FGF2 has been shown to reduce inward remodeling in a mouse model of carotid artery flow cessation.19 The specific function of FGF2 during these remodeling events is not clear. Previous studies suggest that FGF2 could possibly be affecting vascular cell turnover, gene expression, or matrix restructuring in the adapting vessel.20–25

We used a novel mouse model of vessel remodeling, with FGF2 knockout (Fgf2−/−) mice, to test the hypothesis that FGF2 is required during flow-dependent arterial remodeling. The model induces inward (low flow–induced) and outward (high flow–induced) remodeling in the left and right carotid arteries, respectively.

Methods
An expanded Methods section is available online at http://atvb.ahajournals.org.

Experimental Animals
Male FGF2 wild-type (Fgf2+/+) and Fgf2−/− mice26 (50% Black Swiss and 50% 129 SV) were used for all experiments in accordance with University of Arizona Institutional Animal Care and Use–approved procedures. All mice were genotyped by polymerase chain reaction by using primers specific for the FGF2 wild-type and knockout alleles, after collection of genomic DNA. Procedures were timed so that all mice were 8 weeks of age (±4 days) at the time of euthanasia. Mice were anesthetized with 2.5% Avertin (Aldrich) at a dose of 0.15 mL/10 g body wt injected intraperitoneally.

Surgery to Induce Flow-Dependent Carotid Remodeling
The mouse model presented is a modification of procedures previously published for use in the rat.27,28 All branches originating from

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the left common carotid artery (LCCA), except for the left thyroid artery, were ligated (6.0 silk) to reduce flow in the LCCA and increase flow in the contralateral right common carotid artery (RCCA). Mice were euthanized at days 4, 7, and 28 after surgical ligation. Age- and sex-matched control animals were euthanized without having undergone ligation.

**Carotid Artery Blood Flow**

Carotid artery (RCCA and LCCA) blood flow was measured with the use of an ultrasonic transit-time flowmeter (Transonic Systems) with a 0.5-V series probe as described previously. Blood flow was evaluated in mice (n=3 per genotype) before ligation, immediately after ligation, and again at day 14 after ligation.

**Morphometry**

Control (n=4 mice per genotype), day 7 (n=6 mice per genotype), and day 28 (n=7 Fgf2+/+ mice and n=6 Fgf2−/− mice) were perfusion-fixed at constant pressure (90 to 100 mm Hg) with 10% formalin through a polyethylene catheter placed in the left ventricle. The neck, between the clavicle and mandible, was isolated, placed in fixative overnight, and then decalcified by using Decalcifier I and II (Surgipath) for 24 hours each. After paraffin embedding, serial cross sections were cut (8 μm) and stained with hematoxylin. Morphometric analysis was performed on the carotid arteries from 2 whole-neck sections for each animal that were cut 160 to 200 μm apart and located at approximately the midpoint of the common carotid artery.

**Angiography**

For angiography, the arterial circulation was perfused (constant pressure of 90 to 100 mm Hg) with PBS containing 1×10−3 mol/L sodium nitroprusside, followed by filling with contrast agent (210% wt/vol) barium sulfate, Liqui-Coat, Lafayette Pharmaceuticals) through a catheter inserted into the left ventricle. Angiograms of the head and neck region were obtained with the use of a high-definition x-ray cabinet system (Faxitron).

**Vascular Cell Proliferation and Apoptosis**

To examine proliferation, animals (n=3 per genotype) were injected intraperitoneally with bromodeoxyuridine (BrdU, 30 mg/kg body wt; Sigma Chemical Co) at 24 hours and 12 hours before euthanasia on day 4 after LCCA surgery. Mice were perfusion-fixed, and the vertebrae were removed by careful dissection before paraffin embedding. BrdU incorporation into the nuclei of proliferating cells was identified on 6-μm sections with the use of a peroxidase-conjugated sheep anti-BrdU antibody (Biodesign International). BrdU-positive nuclei were counted per 2 whole-vessel transverse sections from each artery. Apoptotic cells were identified by using a Boehringer-Mannheim In Situ Cell Death Detection Kit.

**Statistical Analysis**

Values are presented as mean±SEM. Comparison between 2 means was accomplished by using the Student unpaired t test. Multiple groups were compared by 1-way ANOVA with a Student-Newman-Keuls test. Comparison of carotid artery blood flow, within a genotype, before and after ligation was accomplished by using a 1-way repeated-measures ANOVA with a Student-Newman-Keuls test. Statistical significance was set at P<0.05.

**Results**

**Carotid Artery Blood Flow**

Average blood flow was evaluated in the LCCA and the RCCA (Figure 1; also Table I, please see http://atvb.ahajournals.org). Acutely, the procedure significantly reduced flow in the LCCA (decreased by >80%) and significantly increased flow in the contralateral RCCA (increased by >40%). These changes persisted by day 14 after the LCCA surgery. Blood flow values were not significantly different between Fgf2+/+ and Fgf2−/− mice. Similar to previously published data, ligation of just the left external carotid artery caused only a modest decrease in LCCA blood flow (=30% reduction) and resulted in no change in blood flow within the contralateral RCCA (data not shown).

**Flow-Dependent Arterial Remodeling**

At day 7 and day 28 after surgery, in Fgf2+/+ and in Fgf2−/− mice, the low-flow LCCA was inwardly remodeled, whereas the high-flow RCCA was outwardly remodeled (Figure 2; also Tables II and III and Figure I, please see http://atvb.ahajournals.org). Angiograms of the carotid circulation at day 28 after LCCA surgery show that remodeling appears to take place along the entire length of the common carotid artery (Figure 3). In Fgf2+/+ mice, the diameter changes observed at day 28 corresponded to a 47% decrease in LCCA luminal area and an increase of 33% in RCCA luminal area. Equivalent changes were observed in the day-28 remodeled arteries of Fgf2−/− mice, showing a 50% decrease in LCCA luminal area and an increase of 42% in RCCA luminal area. There were no statistically significant differences in the...
angiography in control mice and at day 28 in the low-flow LCCA of both genotypes (Figure II). However, medial cell density did not change in the day-28 remodeled arteries (Figure 4). The RCCA showed a trend of increased medial cell number at day 28 in both genotypes, but this difference was not significant. No differences were observed between Fgf2+/+ and Fgf2−/− mice.

Discussion
Previous studies have implicated FGF2 in a wide variety of vascular cell-signaling processes, including proliferation, differentiation, and migration (see review33). Specific to flow-dependent remodeling, arterial endothelial cells upregulate Fgf2 mRNA levels in response to fluid shear stress,17 and FGF2 expression increases in the vascular wall during flow-induced arterial enlargement.18 In addition, FGF2 regulates the expression of molecules involved in extracellular matrix remodeling,24 which is an important component of arterial wall reorganization.1,2 Furthermore, FGF2 is also thought to be an important regulator of endothelial cell and smooth muscle cell proliferation22,34–36 and apoptosis.20,21,37 The most direct evidence to date supporting a role for FGF2 in arterial remodeling is the attenuation of inward remodeling by antibody neutralization of endogenous FGF2 in a mouse model of carotid artery flow cessation.19 Despite this large body of evidence, we did not observe an essential role of FGF2 in flow-dependent carotid artery remodeling, given the equivalent remodeling responses of Fgf2+/+ and Fgf2−/− mice. Thus, the results of the present study do not support the

However, there was a noticeable trend toward a reduced diameter in the LCCA and an increased diameter in the RCCA even at this early time point. Perfusion with PBS containing 1×10−3 mol/L sodium nitroprusside to maximally dilate the carotid vessels before fixation demonstrated no diameter differences compared with perfusion with PBS without vasodilator at days 7 and 28 (data not shown). This suggests that the changes in diameter are structural and are not simply alterations in vascular tone. Examination of serial sections showed no intimal lesion formation (neointima) in day-28 mice (Figure 2). A single Fgf2+/+ mouse (and no Fgf2−/− mouse) examined at day 7 (of 6 total mice) had a small intimal lesion. Serials sections showed that the intimal lesion in this mouse was not present along the entire length of the vessel.

Vascular Cell Turnover
Examining the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) index in the medial layer of day-4 remodeled vessels showed increased apoptosis in the RCCA and LCCA. Concomitantly, changes in the BrdU index showed an increased rate of cell proliferation in both remodeled arteries at day 4 (Figure 4). The single intimal cell layer displayed similar changes in cell turnover (data not shown), and positive BrdU and TUNEL staining was observed on the luminal side of the internal elastic lamina at day 4 (Figure III, please see http://atvb.ahajournals.org). To determine the net change in smooth muscle cell number, we counted the total nuclei per medial cross section at day 28. This showed a significantly reduced medial cell count at day 28 in the low-flow LCCA of both genotypes (Figure II). However, medial cell density did not change in the day-28 remodeled arteries (Figure 4). The RCCA showed a trend of increased medial cell number at day 28 in both genotypes, but this difference was not significant. No differences were observed between Fgf2+/+ and Fgf2−/− mice.

Discussion
Previous studies have implicated FGF2 in a wide variety of vascular cell-signaling processes, including proliferation, differentiation, and migration (see review33). Specific to flow-dependent remodeling, arterial endothelial cells upregulate Fgf2 mRNA levels in response to fluid shear stress,17 and FGF2 expression increases in the vascular wall during flow-induced arterial enlargement.18 In addition, FGF2 regulates the expression of molecules involved in extracellular matrix remodeling,24 which is an important component of arterial wall reorganization.1,2 Furthermore, FGF2 is also thought to be an important regulator of endothelial cell and smooth muscle cell proliferation22,34–36 and apoptosis.20,21,37 The most direct evidence to date supporting a role for FGF2 in arterial remodeling is the attenuation of inward remodeling by antibody neutralization of endogenous FGF2 in a mouse model of carotid artery flow cessation.19 Despite this large body of evidence, we did not observe an essential role of FGF2 in flow-dependent carotid artery remodeling, given the equivalent remodeling responses of Fgf2+/+ and Fgf2−/− mice. Thus, the results of the present study do not support the
hypothesis that FGF2 is required for large-artery restructuring in response to chronically decreased or increased blood flow. The apparent contradiction between our results and those of the previous study in which antibodies to FGF2 attenuated inward remodeling after complete LCCA ligation in mice may simply be due to the differences between the 2 models (flow-cessation versus low-flow remodeling) and the distinct stimuli present in each model. In the flow-cessation model, originally published by Kumar and Lindner, net forward blood flow was completely interrupted, resulting in blood stasis in the LCCA. By contrast, our procedures cause substantial blood flow reduction, but forward flow is maintained within the LCCA via the patent thyroid artery. After complete ligation, a gradient of increased intimal lesion formation is observed toward the clotted ligation site. Depending on the strain used, varying amounts of neointimal lesion formation and/or inward remodeling are observed in the ligated artery. Also, it has been reported that the endothelial layer, although intact, detaches from the internal elastic lamina in the ligated LCCA. This exposes the highly thrombogenic extracellular matrix and may increase the activation of blood components, such as platelets. The possibility exists that additional factors, not present in the low-flow situation, contribute to the carotid artery responses induced by complete flow cessation. Platelet activation, hypoxia, metabolite accumulation, and/or inflammation could potentially influence the remodeling response in the completely ligated artery. Such a stimulus, possibly unique to the no-flow condition, may require FGF2 signaling to induce inward remodeling. Alternatively, animals with a chronic gene ablation (eg, knockout mice) and animals with an acute loss of a gene product (eg, antibody neutralization) may simply have different responses to a given stimulus. Last, it is possible that FGF2-neutralizing antibodies are cross-reacting with other FGFs, given that there are at least 23 known FGF family members.

The changes in flow and the resulting carotid remodeling observed in the present study in the mouse are comparable to those in prior studies in the rat but different from a previous study in the mouse in which blood flow was only moderately reduced and the LCCA diameter was decreased by just 8% to 10%. The LCCA branch ligations, performed in the present study, reduced flow in the LCCA by ~80% while increasing the contralateral RCCA flow by 40%. The comparable procedures in the rat reduced LCCA flow by 90% and increased RCCA flow by 45%. These flow changes in the rat caused a 16% reduction in LCCA outer diameter and an 11% increase in RCCA outer diameter after 4 weeks of remodeling. In comparison, wild-type mice in the present study showed an ~23% reduction in LCCA vessel diameter and a 13% increase in RCCA vessel diameter at day 28. In rabbits, ligation of the left external carotid artery decreased LCCA blood flow by 70%, causing a 21% reduction of LCCA luminal diameter after 2 weeks. Overall, our results in the mouse are consistent with previous studies of flow-dependent remodeling in other species, demonstrating that chronically increased blood flow leads to arterial enlargement (outward remodeling), whereas blood flow reduction results in arterial narrowing (inward remodeling).

Figure 4. A, Quantification of medial cell proliferation (n=3 per genotype) for control and day-4 remodeled LCCA and RCCA. B, Quantification of medial cell apoptosis (n=3 per genotype) for control and day-4 remodeled arteries. C, Medial cell density for control and day-28 remodeled arteries. *P<0.05 vs respective control; P=NS for Fgf2+/+ vs Fgf2−/−.

Associated with structural remodeling in this model is increased vascular cell turnover, as indicated by increased apoptosis and proliferation in the low-flow LCCA and the high-flow RCCA early in the remodeling process. Previous investigators have observed increased BrdU labeling and increased apoptosis in vessels after chronic flow reduction. Similar to others, we demonstrate a net loss of vascular cells after chronic flow reduction in the mouse. Specifically, we observed a reduced medial smooth muscle cell count in the low-flow LCCA at day 28. However, medial smooth muscle density remained unchanged because of the noticeable trend toward reduced medial CSA in the LCCA. In the high-flow RCCA, apoptosis and proliferation increased, but there was not a significant change in medial cell count, density, or CSA compared with control conditions. A study of...
flow remodeling in the rat mesentery showed that increased apoptosis and proliferation occurred simultaneously in high-flow exposed resistance arteries and that this was coupled to an increase in medial CSA of high-flow arteries. Miyashiro et al. showed no change in medial CSA in the low-flow carotid artery of juvenile rats, whereas the high-flow carotid artery had increased medial CSA. Taken as a whole, the present results are in agreement with the concept that chronic changes in blood flow result in dynamic changes in vascular cell turnover, although we observed a constancy in medial cell density in both flow conditions.

The specific molecules regulating vascular cell growth or apoptosis during flow remodeling are largely unknown. Carotid arteries of endothelial NO synthase (eNOS) knockout (eNOS’s−/−) mice subjected to chronically reduced flow showed increased vascular cell proliferation and cell number compared with carotid arteries of eNOS wild-type (eNOS’s+/+) mice. Thus, endothelium-derived NO may be an essential controller of vascular cell turnover during flow-dependent carotid remodeling. Previous studies show that FGF2 can mediate endothelial cell and smooth muscle cell proliferation and apoptosis. Interestingly, FGF2 has been shown to stimulate eNOS mRNA expression and eNOS protein production in cultured endothelial cells. Also, it has been shown that NO promotes proliferation of in vitro endothelial cells through endogenous FGF2. However, there was no difference between Fgf2’s−/− and Fgf2’s−/+ mice in the present study that would indicate that vascular cell turnover was affected by lack of FGF2. Thus, FGF2 does not appear to be an essential mediator acting upstream or downstream of NO signaling in this model. In terms of vascular cell proliferation, it has previously been shown that the carotid arteries of Fgf2’s−/− mice undergo a normal hyperplastic response after intra-arterial mechanical injury. Also, intimal area and cellularity were not affected by FGF2 antibody in the ligated mouse carotid artery, suggesting that smooth muscle proliferation was not altered by FGF2 neutralization.

Overall, the apparently normal remodeling responses observed in Fgf2’s−/− mice may reflect compensation for the loss of FGF2 by another gene product. There are numerous FGF family members, and these proteins bind to a common group of receptors, although with differing affinities. Thus, it is possible that at least one FGF protein could be compensating for the disruption of the Fgf2 gene. Recently, a double knockout of FGF1 and FGF2 was shown to have the same phenotype as Fgf2’s−/− mice. This suggests that FGF1, the FGF family member most closely related to FGF2, is not compensating for the loss of FGF2 in situations such as development and wound healing. On the other hand, it is possible that there is not compensation and that other growth factors or molecules may be the actual endogenous mediators of processes currently ascribed to FGF2 (eg, flow-dependent remodeling). In this regard, changes in FGF2 expression may be mediating some other event during arterial remodeling that is either unrelated or not critical for structural changes in the artery.

It is important to note that considerable strain variability in the vascular responses of mice to various challenges has been described. More specifically, Harmon et al. using the LCCA flow-cessation model in mice, demonstrated a large degree of strain-dependent variability in carotid remodeling of the ligated LCCA. Additionally, they showed that not all strains displayed significant outward remodeling of the contralateral RCCA. Others using the flow-cessation model observed no RCCA enlargement despite measuring a near doubling of RCCA blood flow in 129 SV mice. Thus, it is reasonable to expect that there might be strain-specific differences in the extent and character of vessel remodeling when the model presented in the present study is used. In preliminary experiments, we noted that the LCCA and RCCA of FVB/NJ and C57BL/6J mice had been remodeled to an extent similar to that observed in Fgf2’s−/− and Fgf2’s−/+ mice, which are on a mixed background of 50% Black Swiss and 50% 129 SV (data not shown). These other strains showed inward remodeling with minimal neointimal lesion formation in the LCCA. When a neointima was observed in these mice, it was typically only 2 or 3 cell layers thick (data not shown). We also noticed strain-dependent variations in the carotid artery architecture (ie, position of branching vessels).

In conclusion, we describe a model of bilateral carotid remodeling in the mouse. In a single mouse, the simultaneous reduction in blood flow in the LCCA and increase in blood flow in the RCCA provide a powerful research tool to effectively examine the molecular mechanisms of artery remodeling. With this model, we show that lack of FGF2 does not affect structural remodeling of large arteries in response to chronically altered blood flow. FGF2 appears dispensable during flow-dependent remodeling of the artery wall and does not significantly regulate vascular cell turnover in this model.

Acknowledgments

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References


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Online Only Data Supplement (Tables I, II, & III)

Table I. Blood flow changes before and after ligation of LCCA branches in $Fgf2^{+/+}$ and $Fgf2^{-/-}$ mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Day 0</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>$Fgf2^{+/+}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(n)$</td>
<td>0.77 ± 0.02</td>
<td>0.74 ± 0.04</td>
<td>1.11 ± 0.05*</td>
</tr>
<tr>
<td>$Fgf2^{-/-}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(n)$</td>
<td>0.73 ± 0.05</td>
<td>0.73 ± 0.07</td>
<td>1.03 ± 0.01*</td>
</tr>
</tbody>
</table>

Average blood flow (ml/min) in the LCCA (left) and RCCA (right) of $Fgf2^{+/+}$ and $Fgf2^{-/-}$ mice before ligation of LCCA branches, immediately after the ligations (day 0), and again at 14 days after the ligations (day 14). Values are mean ± SEM *P<0.05 vs respective control (before) values. $Fgf2^{+/+}$ vs $Fgf2^{-/-}$, not significantly different.
Table II. Time course of arterial remodeling in Fgf2+/+ mice following ligation of the left common carotid artery (LCCA) branches.

<table>
<thead>
<tr>
<th>Fgf2+/+</th>
<th>Control</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 28</th>
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</thead>
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<td></td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>(4)</td>
<td>(8)</td>
<td>(6)</td>
</tr>
<tr>
<td>IEL Perimeter (µm)</td>
<td>961 ± 14</td>
<td>957 ± 8</td>
<td>990 ± 28</td>
<td>855 ± 35</td>
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<tr>
<td>EEL Perimeter (µm)</td>
<td>1073 ± 21</td>
<td>1069 ± 15</td>
<td>1099 ± 26</td>
<td>1018 ± 38</td>
</tr>
<tr>
<td>Lumen Diameter (µm)</td>
<td>306 ± 4</td>
<td>305 ± 3</td>
<td>315 ± 9</td>
<td>270 ± 11</td>
</tr>
<tr>
<td>Vessel Diameter (µm)</td>
<td>342 ± 7</td>
<td>340 ± 5</td>
<td>350 ± 8</td>
<td>324 ± 12</td>
</tr>
<tr>
<td>Lumen Area (µm²)</td>
<td>73460 ± 2150</td>
<td>72870 ± 1258</td>
<td>78384 ± 4460</td>
<td>58879 ± 4605</td>
</tr>
<tr>
<td>Vessel Area (µm²)</td>
<td>91789 ± 3517</td>
<td>90959 ± 2627</td>
<td>96554 ± 4728</td>
<td>83217 ± 6012</td>
</tr>
<tr>
<td>Medial Area (µm²)</td>
<td>18328 ± 1371</td>
<td>18089 ± 1454</td>
<td>18170 ± 621</td>
<td>24339 ± 3118</td>
</tr>
<tr>
<td>Medial Thickness (µm)</td>
<td>18 ± 1</td>
<td>18 ± 1</td>
<td>17 ± 1</td>
<td>26 ± 3</td>
</tr>
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Table III. Time course of arterial remodeling in Fgf2/- mice following ligation of the left common carotid artery (LCCA) branches.

<table>
<thead>
<tr>
<th>Fgf2-/− (n)</th>
<th>Control</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right (4)</td>
<td>Left (4)</td>
<td>Right (8)</td>
<td>Left (8)</td>
</tr>
<tr>
<td>IEL Perimeter (µm)</td>
<td>931 ± 19</td>
<td>949 ± 35</td>
<td>985 ± 19</td>
<td>847 ± 28</td>
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<tr>
<td>EEL Perimeter (µm)</td>
<td>1033 ± 18</td>
<td>1052 ± 34</td>
<td>1091 ± 17</td>
<td>1022 ± 37</td>
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<tr>
<td>Lumen Diameter (µm)</td>
<td>296 ± 6</td>
<td>302 ± 11</td>
<td>314 ± 6</td>
<td>269 ± 9</td>
</tr>
<tr>
<td>Vessel Diameter (µm)</td>
<td>329 ± 6</td>
<td>334 ± 11</td>
<td>347 ± 6</td>
<td>325 ± 12</td>
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<tr>
<td>Lumen Area (µm²)</td>
<td>68979 ± 2737</td>
<td>71870 ± 5201</td>
<td>77372 ± 2917</td>
<td>57441 ± 3628</td>
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<td>Vessel Area (µm²)</td>
<td>85004 ± 3022</td>
<td>88325 ± 5642</td>
<td>94794 ± 3010</td>
<td>83883 ± 6288</td>
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<td>Medial Area (µm²)</td>
<td>16025 ± 342</td>
<td>16545 ± 696</td>
<td>17422 ± 573</td>
<td>23602 ± 2258</td>
</tr>
<tr>
<td>Medial Thickness (µm)</td>
<td>16 ± 1</td>
<td>17 ± 1</td>
<td>17 ± 1</td>
<td>25 ± 2</td>
</tr>
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</table>
Figure I. Representative photomicrographs of carotid artery cross-sections from Fgf2+/+ mice (A) Fgf2−/− mice (B) showing control and day 28 flow remodeled arteries (RCCA and LCCA) stained with hematoxylin. Right and left images are from the same histology section (i.e., a single cross-section from an individual animal). Scale bar equals 100µm.
Figure II. A, Medial cell count, equal to number of nuclei per medial cross section, for control and day 28 remodeled LCCA and RCCA. B, Medial area (cross-sectional) calculated for control and day 28 remodeled arteries. *P<0.05 vs respective control. Fgf2+/+ vs Fgf2−/−, not significantly different.
Figure III. Representative photomicrographs of carotid artery cross sections at 4 days after ligation of LCCA branches stained for TUNEL (apoptosis) or BrdU (proliferation) and counterstained with hematoxylin. Scale bar equals 25µm.
MATERIALS AND METHODS (Online Supplement)

Experimental animals

Male $Fgf2^{+/+}$ and $Fgf2^{-/-}$ mice (50% Black Swiss and 50% 129 SV) were used for all experiments according to the University of Arizona IACUC approved procedures. All mice were genotyped by PCR using primers specific for the $Fgf2$ wildtype allele (forward, 5’–GCTGTACACTCAAGGGGCTC – 3’; reverse, 5’– CGCCGTTCTTGACGAG – 3’) and the $Fgf2$ knockout allele (forward, 5’– TCCAAAGCCTGACTTGATCC – 3’; reverse, 5’–CTGACTAGGGGGAGGAGTAGAAGG – 3’), following collection of genomic DNA from tail clips. Procedures were timed so that all mice were 8 weeks of age (± 4 days) at the time of sacrifice. Mice were anesthetized with 2.5% Avertin (2.5% 2,2,2-tribromoethanol, 2.5% tert-amyl alcohol in PBS: Aldrich) at a dose of 0.15 ml per 10 gram body weight injected intraperitoneally.

Surgery to induce flow-dependent carotid remodeling

The mouse model presented is a modification of procedures previously published for use in the rat. Mice were anesthetized and a midline incision was made along the neck. All branches originating from the left common carotid artery (LCCA), except for the left thyroid artery, were ligated (6.0 silk) in order to reduce flow in the LCCA and increase flow in the contralateral right common carotid artery (RCCA). Specifically, the distal portion of the left external carotid was ligated where it bifurcates while the internal carotid and occipital arteries were ligated with a single suture at their origin (Figure 1). Mice were sacrificed at day 4, 7, and 28 following
surgical ligation. Age and sex matched control animals were sacrificed without having undergone ligation.

**Carotid artery blood flow**

Carotid artery (RCCA and LCCA) blood flow was measured using an ultrasonic transit-time flowmeter (Transonic Systems, Inc.) with a 0.5 V-series probe as described previously. Blood flow was evaluated in mice (n=3 per genotype) before ligation, immediately after ligation (day 0), and again at day 14 after ligation. Average volume flow (ml/min) was recorded for 5 min in each artery. The values presented are the average for the 5 min data acquisition period. After ligation of the LCCA branches, blood flow measurements were repeated after a stabilization period of at least 15 min. The incision was closed and mice were allowed to recover. After 14 days, the mice were reanesthetized and blood flow was evaluated for a final time.

**Morphometry**

Control (n=4 per genotype), day 7 (n=6 per genotype), and day 28 (+/+ n=7; -/- n=6) animals were perfuse fixed through a polyethylene catheter placed in the left ventricle. Animals were perfused at constant pressure (90-100 mmHg) with 20 ml of heparinized PBS followed by 10 ml of 10% phosphate buffered formalin. The neck, between the clavicle and mandible, was isolated and placed in fixative overnight. Neck sections were then decalcified using Decalcifier I and II (Surgipath) for 24 hr each. Decalcified necks were processed, paraffin embedded, and serial sections cut (8 µm) for morphometric analysis. Cross sections of the entire neck were stained using hematoxylin. Morphometric analysis was carried out on the RCCA and LCCA of each animal from 2 whole neck cross sections approximately 160-200 µm apart, cut at approximately
the mid-portion of the common carotid artery. Digitized images were analyzed using image analysis software (Scionimage 4.0). The perimeter (length) of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were measured and these values were used to calculate various vessel dimensions, assuming the artery was a perfect circle.⁵,⁶

**Angiography**

Mice were anesthetized and subsequently overdosed with 2.5% Avertin following exposure of the thoracic cavity and heart. The arterial circulation was perfused (constant pressure of 90-100 mmHg) with PBS containing 1x10⁻⁵ mol/L sodium nitroprusside through a tapered polyethylene catheter (PE-90) inserted into the left ventricle followed by filling with contrast agent (barium sulfate 210% w/v, Liqui-Coat, Lafayette Pharmaceuticals, Inc). Angiograms of the head and neck region were obtained using a high-definition x-ray cabinet system (Faxitron).

**Vascular cell proliferation and apoptosis**

To examine proliferation, animals (n=3 per genotype) were injected with bromodeoxyuridine (BrdU: 30mg/kg body weight: Sigma Chemical Co., St.Louis, MO) i.p. at 24 h and 12 h prior to sacrifice on day 4 following LCCA surgery. Decalcification interfered with BrdU and TUNEL staining. So, mice were perfuse fixed and the vertebrae along with all other bones were removed by careful dissection prior to paraffin embedding. BrdU incorporation into the nuclei of proliferating cells was identified on 6-µm sections⁷ using a peroxidase conjugated sheep anti-BrdU antibody (Biodesign International, Kennebunk, ME). BrdU-positive nuclei were counted per two whole vessel transverse sections from each artery. Proliferation is expressed as the percentage of the total nuclei counted in the vessel cross-section that stain positive for BrdU.
Apoptotic cells were identified using Boehringer-Mannheim’s In Situ Cell Death Detection Kit. Apoptosis in each vessel was expressed as a percentage of total nuclei per vessel cross-section that are labeled as apoptotic. Calculations (e.g., proliferation index) were performed for the media (between the IEL and EEL), intima (lumen side of the IEL), and the whole vessel (intima + media).

**Statistical analysis**

Values are presented as the mean±SEM. Comparison between two means was done using Student’s unpaired t-Test. Multiple groups were compared by One Way ANOVA with a Student-Newman-Keuls Test. Comparison of carotid artery blood flow, within a genotype, before and after ligation was done using a One Way Repeated Measures ANOVA with a Student-Newman-Keuls Test. Statistical significance was set at $P < 0.05$.

**REFERENCES**


