Coronary collateralization or arteriogenesis is a vascular adaptive remodeling process that occurs in the context of arterial occlusion when preexisting collateral arterioles remodel to form larger diameter bypass arteries. The current arteriogenesis paradigm implicates local hemodynamics, recruitment/activation of monocytes, and structural remodeling of the vascular wall as key steps in collateralization (1). Two primary stages of collateralization have been described based predominantly on observations from femoral artery ligation models that induce collateral remodeling via an instantaneous increase in collateral flow. The initial stage of collateralization is characterized by a wave of monocyte-driven inflammation and rapid vasodilation, an immediate compensatory response to increased collateral flow. In the second phase of collateralization, the initial inflammatory response is resolved, and the newly formed conductance artery wall is stabilized (2). To date, considerable attention has been dedicated to investigating the inflammatory regulation of this process (i.e. MCP-1 and GM-CSF), but despite evidence on the importance of the endothelium and endothelium-derived factors in flow-mediated arterial remodeling (3–5), less is known about how altered collateral vessel shear stress acting on the endothelium affects collateralization. Characterizing the specific role collateral flow plays in regulating functional endothelial cell phenotypes, and endothelium-dependent processes associated with adaptive remodeling should therefore provide a framework to better understand the establishment, maintenance, and remodeling of arteries.

A number of signaling molecules have previously been characterized for their role in controlling smooth muscle cell phenotypes in both blood vessel maturation and vascular remodeling. For example, mice lacking platelet-derived growth factor B, S1P1 (sphingosine-1 phosphate-1), or KLF2 (Kruppel-like factor 2) are embryonic lethal due to defects in vascular wall formation. For example, mice lacking platelet-derived growth factor B, S1P1 (sphingosine-1 phosphate-1), or KLF2 (Kruppel-like factor 2) are embryonic lethal due to defects in vascular wall formation and function (6–11). Models of arterial injury have also characterized for their role in controlling smooth muscle cell proliferation and migration, and evidence exists indicating that the endothelium contribu-
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Lumped Parameter Numerical Simulation of Collateral Vessel Shear Stress Waveforms—A lumped parameter hemodynamic model was created to numerically simulate shear stress waveforms acting along the no stenosis/normal coronary collateral (NCC) and partial stenotic/adaptive remodeling coronary collateral vessels (ACC). A number of assumptions were made for the simulations based on what is currently known regarding the geometric parameters of the coronary collateral circulation. First, a bridging lesion geometry was assumed in which the coronary collateral vessels originate proximally and terminate distally to the site of occlusion in the same vessel (see parallel vessel segments in Fig. 1, A and B). This common collateral geometry has been observed in greater than 20% of patients with developed coronary collaterals (26). Next, since both the collateral size and distribution (i.e. number of collateral vessels bypassing a stenosis) have been demonstrated to vary in patients and between sites of stenosis (26), we assumed a predeveloped collateral diameter of 200 μm, which is consistent with human arterioles, with flow distributed across 10 parallel collaterals. Last, a concentric, 60% coronary artery stenosis (radius reduction) was assumed based on the clinical range of a critical stenosis and what is feasible to reproduce experimentally (27).

With boundary pressure tracings obtained from previously published data, fourth order Runge-Kutta numerical integration (Matlab; Mathworks) was used to solve for the individual time-varying nodal pressure tracings (see supplemental material) (28). These results were then used to calculate the instantaneous pressure difference across each vessel segment, ΔP, for both the NCC and ACC simulations, and subsequently the wall shear stress τ acting along the collateral vessel by the equation,

\[ \tau = \frac{r \Delta P}{2L} \]  

(Eq 1)

where \( r \) represents the vessel radius, and \( L \) is the vessel length (see supplemental material for detailed description of the simulation calculations).

Endothelial Cell Culture—Primary human umbilical vein EC were isolated, cultured, and subjected to shear stress waveforms, as previously described (29). Reduced serum and growth factor medium was used for the shear experiments (Medium-199, supplemented with 2% fetal bovine serum, 2 mm l-glutamine, 100 units/ml penicillin plus 100 μg/ml streptomycin, and 2.8% dextran (Invitrogen)) with a viscosity of 2.2 Centipoise (0.0022 Pascal/seconds). Shear stress application to EC using the NCC and ACC shear stress waveforms was performed using a dynamic flow system (29). For the induction of KLF2 expression, EC were plated at a cell density of 60,000 cells/cm², and the next day they were infected with a KLF2-GFP adenovirus (EC-KLF2) or a control GFP adenovirus (EC-GFP) at a multiplicity of infection of 20. Alternatively, for the connective tissue growth factor (CTGF) luciferase activity assays, EC were plated at a density of 20,000/cm² and grown overnight. The next day, cells were simultaneously transfected with 1.4 μg of a luciferase construct (pGL3 basic; Promega) driven by ~2.0 kb of the CTGF proximal promoter (~2065 to +72 bp, provided by Dr. Eugene Chen, University of Michigan (30)) and 0.6 μg of β-galactosidase, for normalization, using FuGene6 (Roche Applied Science). 24 h later, the EC were infected with the KLF2 or control adenovirus as described above. After an additional 24 h, the CTGF promoter activity was analyzed.

Smooth Muscle Cell Culture and Delivery of Conditioned Medium—Human aortic vascular SMC (CRL-1999; ATCC) were grown and propagated in complete growth medium according to the supplier’s instructions. For experiments in which conditioned medium containing flow-mediated endothelium-derived factors was continuously delivered to SMC, 100,000 SMC were plated on 0.1% gelatin (Difco)-coated glass coverslips (25 × 75 mm, number 1; Belco) 24 h prior to exposure to conditioned medium and maintained in serum-free medium (complete growth medium with the 10% fetal bovine serum removed) at 37 °C and 5% CO₂. On the day of experimentation, ACC and NCC shear stress waveforms imposed on EC were initiated in the dynamic flow system 1 h prior to connecting to the downstream SMC culture chamber via 1% bovine serum albumin (BSA; Sigma)-coated Tygon tubing. "No endothelium" control experiments were conducted with SMC grown and maintained in the same culture chamber but in the absence of upstream EC.

Transcriptional Profiling—Labeling, hybridization, and scanning of microarrays for ACC versus NCC flow-stimulated EC transcriptional profiling were performed according to the manufacturer’s protocols for the ABI7000 microarray system using total genome microarrays containing 30,096 spots representing 28,790 distinct genes (Applied Biosystems) and as previously described (31). The microarray raw signal values were filtered...
for signal/noise quality and normalized using Lowess regression. Differential regulation of NCC and ACC flow-mediated genes was then analyzed in custom-designed Matlab (Mathworks) software for significance using three replicate experiments and Z-pool statistical methodology, defining statistically significant gene regulation for \( p < 0.001 \) (32). Differentially regulated genes were then sorted by biological function using the publicly available Panther data base (available on the World Wide Web; Applied Biosystems) and cross-referenced with the Gene Ontology classification (available on the World Wide Web). The microarray data were submitted as a data set series to the Gene Expression Omnibus (GEO) (accession number GSE11583).

RNA Preparation and Reverse Transcription-PCR Analysis—Total RNA from EC and SMC used for real-time PCR was isolated using Lysis Buffer (Applied Biosystems) and purified using the Prism Nucleic Acid Prep-Station (Applied Biosystems) according to the manufacturer’s instructions. RNA quantity was measured by spectrophotometric analysis at 260 nm, and quality was verified by an Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip kit. Purified, DNase-treated RNA (0.375 \( \mu \)g) was reverse-transcribed using a MultiScribe-based 25-\( \mu \)l reaction (Applied Biosystems). The cDNA was diluted in 50 \( \mu \)l of DNase-free water and subjected to a 20-\( \mu \)l real time TaqMan quantitative PCR (Applied Biosystems 7900).

Microfluidic Device Fabrication—The microfluidic system was made of polydimethyl siloxane (Silgard 184; Dow Chemical) using soft lithography with SU-8 patterned wafers as described by others (33). The fabricated devices were autoclaved and then plasma-treated for 60 s (Harrick) in air and bonded with sterile number 1 circular glass coverslips (VWR) to form closed microfluidic channels. The bonded devices were maintained at 80 °C for 10 min to enhance bonding strength, and then a 0.1% poly-D-lysine (Sigma) coating solution was introduced into the channels and incubated at 37 °C and 5% CO\(_2\) overnight. After coating, the devices were washed twice with sterile water and dried at 80 °C for 24 h, rendering the channel surfaces hydrophobic. Scaffold material, 0.25% type I collagen (BD Biosciences), was injected into the three-dimensional device region and incubated for 30 min at 37 °C in order to form a three-dimensional hydrogel scaffold for cell invasion. Once the scaffold formed, 0.1% gelatin was then introduced into the microfluidic channels and incubated at 37 °C and 5% CO\(_2\) for 60 min to make the channel suitable for cell seeding. The gelatin was then replaced by cell culture medium and allowed to equilibrate for 2 h at 37 °C at 5% CO\(_2\) before cell seeding.

Three-dimensional Migration Assay Cell Seeding and Quantification—24 h after adenoviral infection, EC were seeded via one channel of the three-dimensional microfluidic device (\( \mu \)FD) at a density of 2 \( \times \) 10^5 cells/ml in complete endothelial growth medium–2 (EGM-2; Cambrex) and maintained for 24 h at 37 °C at 5% CO\(_2\), 2 h prior to SMC seeding. EGM-2 in the microfluidic device was exchanged for co-culture medium (Medium-199, supplemented with 2% fetal bovine serum, 2 mm \( L \)-glutamine, 100 units/ml penicillin plus 100 \( \mu \)g/ml streptomycin, and 0.03 mg/ml endothelial cell growth supplement) in order to allow the three-dimensional region to equilibrate with the co-culture medium. SMC were subsequently labeled with the fluorescent membrane stain PKH26 (Sigma) according to the manufacturer’s instructions and seeded in the channel opposing the endothelial channel at a density of 500,000 cells/ml. The EC/SMC co-culture was then maintained at 37 °C and 5% CO\(_2\) for 48 h before quantification and analysis of SMC migration. For connective tissue growth factor (human recombinant CTGF; Invitrogen) migration studies, the devices were fabricated, and the collagen gel was introduced as described above. However, SMC were seeded on the same day as collagen hydrogel injection following 0.1% gelatin coating directly in the co-culture medium described above.

SMC three-dimensional invasion for both co-culture and CTGF migration experiments was quantified by staining cell nuclei with DAPI after 48 h of migration. Briefly, the cells were fixed in 4% paraformaldehyde for 60 min at room temperature, permeabilized with 1% Triton-X for 45 min at room temperature, and DAPI-stained overnight at 4 °C in phosphate-buffered saline containing 0.1% fetal calf serum. Fluorescent images of cell nuclei and PKH26-stained SMC were then obtained for the entire three-dimensional hydrogel region using an inverted epifluorescent microscope (Nikon Eclipse TE2000-U) and a CCD camera (Sanyo VCB-3524). By merging the two images of different wavelength, nuclei of the prelabeled SMC could be distinguished from the EC, along with the coordinate position of each SMC within the three-dimensional hydrogel (MetaMorph). The distribution of cell number versus three-dimensional invasion/migration distance and total number of invading cells after 48 h of co-culture was quantified using this tracking approach.

Gel Shift Studies—To perform the gel shift studies, a nonradioactive LightShift™ chemiluminescent electrophoretic mobility shift assay kit (Pierce) was used according to the manufacturer’s instructions. Briefly, multiple 5′-biotin-labeled 25-mer DNA oligonucleotide sense and antisense strands were generated from sequences of the CTGF proximal promoter (~2 kb) that encompass the canonical CACCC KLF binding sites (34), with three of the five strands containing a single CACCC site and two of the strands containing two sites in tandem. The sense strands generated were as follows: –119 to –95 (5′-biotin-agggcagaaagttggaggagtaagctc-3′); –844 to –820 (5′-biotin-tgtttcaccctgctacccagaggg-3′); –1085 to –1061 (5′-biotin-tcataagggtttggaggactaa-3′); –1188 to –1164 (5′-biotin-cattgcacccacagggagga-3′); and –1730 to –1706 (5′-biotin-cagggagggaccccaccccttgc-3′). The sense and antisense strands were annealed to generate double-stranded, biotin-labeled probes. To perform the electrophoretic mobility shift assay, 80 fmol of biotin-labeled probes were incubated with 80 ng of purified human KLF2 protein fused to glutathione S-transferase (GST-KLF2; C-terminal amino acids 265–356 with stop codon at amino acid 356), 50 \( \mu \)g/ml poly(dl-dC), 2.5% glycerol, 0.05% Nonidet P-40, 5 mm MgCl\(_2\) in the presence of binding buffer (10 mm Tris-HCl, pH 7.5, 50 mm KCl, 1 mm dithiothreitol) for 20 min at room temperature. After incubation, the DNA–protein complexes were separated by 5% nondenaturing polyacrylamide gel electrophoresis (Bio-Rad) and transferred to a positively charged nylon membrane (Pierce). After UV cross-linking, detection was performed with streptavidin-
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**Statistical Analysis**—Statistical significance was determined by one-way analysis of variance for experiments with more than two subgroups, whereas post hoc and pairwise comparisons were performed by two-tailed Student’s t test for all others (Primer of Biostatistics). p values less than 0.05 were considered significant. Statistical significance for the microarray data is described under “Transcriptional Profiling.”

**RESULTS**

**Hemodynamic Simulation of Coronary Collateral Shear Stress Waveforms**—A lumped parameter model was developed to compute the shear stress waveforms imposed on coronary collateral endothelium (Fig. 1, A and B). The goal of the model was to numerically simulate collateral vessel shear stress waveforms in the normal coronary collateral (designated NCC) when there is no stenosis and in the adaptive remodeling coronary collateral (designated ACC), corresponding to a 60% radius reduction of the main coronary artery. Assigning previously published (35) or fluid dynamically estimated parameters (i.e. vascular resistance (R) and compliance (C)) to each vessel segment of the coronary circulation and applying the corresponding pressure tracing, P(t), boundary conditions (28) (see “Experimental Procedures” and supplemental material) to the NCC and ACC models yielded two distinct pulsatile collateral vessel waveforms (Fig. 1, C and D, respectively). These simulations represent two previously undefined collateral waveforms that provide an estimate of the biomechanical shear stress experienced by both a normal collateral vessel and a collateral vessel undergoing adaptive remodeling.

**Collateral Shear Stress Regulates Endothelial Gene Expression Patterns**—The two simulated coronary collateral waveforms were next applied to cultured human umbilical vein EC for 24 h, and differences in the resulting EC molecular phenotype were determined by genome-wide transcriptional profiling. In total, 681 genes were found to have statistically significant regulation, as determined by Z statistics (32) (Fig. 2A). Further analysis revealed the differential regulation of multiple endothelial genes that have previously been shown to influence both acute and chronic vascular adaptations in the developing embryo and the adult (Table 1). This collateral flow-mediated endothelial gene expression pattern included the differential regulation of vasoactive, angiogenic, inflammatory, extracellular matrix, developmental, secreted, and transcription factor genes. Specifically, ACC flow up-regulated expression of vasodilatory genes (ENOS (endothelial nitric-oxide synthase) and VIP (vasoactive intestinal peptide)), endothelial receptors and ligands required for arterial development (TIE2 (endothelial tyrosine kinase receptor) and JAG1 (tagged 1)), extracellular matrix regulators (NOV (nephroblastoma-overexpressed) and TIMP3 (tissue inhibitor of metalloproteinase 3)), and transcription factors (KLF2, KLF10, KLF13, and FOXF1 (forkhead box f1)). Conversely, ACC flow down-regulated post hoc (tissue inhibitor of metalloproteinase) and (forkhead box f1)).

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Collateral Flow Acting on the Endothelium Regulates Smooth Muscle Cell Gene Expression—In order to determine whether the simulated collateral flow waveforms acting on EC have paracrine ability to differentially regulate smooth muscle gene expression, we developed a system that supports the culture of human aortic smooth muscle cells (SMC) while receiving flow-mediated EC conditioned medium (see supplemental material). The SMC culture system was continuously perfused throughout the duration of the collateral flow experiments and did not allow direct contact between EC and SMC, ruling out effects mediated by cell-cell interactions. With this system, exposure of SMC to ACC flow-mediated EC conditioned medium for 24 h resulted in increased SMC expression of MYOCARD (myocardin), a transcriptional co-activator essential for the expression of multiple SMC contractile proteins (36, 37) (Fig. 2C), and a decrease in SMC expression of MMP-3 (matrix metalloproteinase 3), a matrix-degrading enzyme whose expression is associated with pathologic changes to the vessel wall (Fig. 2D) (38, 39), compared with SMC exposed to NCC flow-mediated EC conditioned medium or no endothelium control medium. Importantly, CNN1 (calponin) and oSMA (α-smooth muscle actin), two SMC genes that are downstream transcriptional targets of myocardin (36), were also increased in SMC exposed to ACC, compared with NCC, flow-mediated EC conditioned medium (Fig. 2E). Thus, by coupling the simulated collateral waveforms with dynamic EC-SMC co-culture, these results demonstrate that collateral flow acting on EC has the capacity to modulate the expression of SMC genes and suggest that flow acting on the endothelium is an important determinant of vascular SMC phenotype during adaptive remodeling.

Collateral Flow-mediated EC Genes Control SMC Migration—SMC migration is a critical event during embryonic blood vessel formation and adult vascular disease processes, such as atherosclerosis, restenosis, and arterial bypass vein grafting in response to changes in the vessel microenvironment (36, 40). Accordingly, we next sought to characterize how endo-

**TABLE 1**

| RefSeq          | Gene name/symbol                      | -Fold change | Remodeling function                      |
|-----------------|---------------------------------------|--------------|------------------------------------------|
| **Up**          |                                       |              |                                          |
| NM_002514.2     | Nephroblastoma overexpressed gene; NOV | 26.5472      | Matricellular protein; ECM protein       |
| NM_194435.1     | Vasoactive intestinal peptide; VIP    | 4.7390       | Vasostriction; secreted peptide          |
| NM_016270.1     | Kruppel-like factor 2 (lung); KLF2    | 4.5575       | Vasostrictive factor                     |
| NM_003662.3     | Tissue inhibitor of metalloproteinase 3; TIMP3 | 3.7026     | Anti-angiogenesis; ECM remodeling        |
| NM_006003.3     | Nitric-oxide synthase 3 (endothelial cell); ENOS | 2.9320    | Vasostriction                           |
| NM_004591.9     | Tyrosine kinase, endothelial; TEK/TIE2 | 2.6057       | Vascular development; receptor           |
| NM_002214.1     | Jagged 1 (Alagille syndrome); JAG1     | 2.2904       | Vascular development; Notch ligand       |
| NM_002006.3     | Fibroblast growth factor 2 (basic); FGF2 | 2.2724     | Angiogenesis/arteriogenesis; secreted    |
| **Down**        |                                       |              |                                          |
| NM_003873.2     | Neuropilin 1; NR1P                   | −2.1897      | Angiogenesis; secreted                   |
| NM_003239.1     | Transforming growth factor, β2; TGFβ2 | −2.1517      | Wound healing; secreted                  |
| NM_001554.3     | Cysteine-rich, angiogenic inducer, 61; CYR61 | −2.2162      | Angiogenesis; secreted                   |
| NM_130851.1     | Bone morphogenetic protein 4; BMP4   | −2.5047      | Inflammation; secreted                   |
| NM_006080.1     | Semaphorin 3A; SEMA3A                | −2.5114      | Angiogenesis; secreted                   |
| NM_001200.1     | Bone morphogenetic protein 2; BMP2   | −2.5437      | Inflammation; secreted                   |
| NM_001901.1     | Connective tissue growth factor; CTGF | −2.6762      | Wound healing; secreted                  |
| NM_001955.2     | Endothelin 1; EDN1                   | −3.0242      | Vasocostriction; secreted                |
| NM_000584.2     | Interleukin 8; IL8                   | −3.5467      | Inflammation; cytokine                   |
| NM_006329.2     | Fibulin 5; FBLN5                     | −3.6664      | Angiogenesis; ECM protein                |
| NM_002983.2     | Chemokine (C-C motif) ligand 2; CCL2 (MCP-1) | −4.6387      | Inflammation; cytokine                   |
| NM_001147.1     | Angiopoietin 2; ANGPT2               | −9.9658      | Angiogenesis; secreted                   |

*Underlined genes are previously defined transcriptional targets of KLF2 (31).

**ECM, extracellular matrix.**
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FIGURE 3. EC expressing KLF2 inhibit EC-directed SMC migration. A and B, a three-dimensional μFD assay was designed in order to investigate SMC migration in response to endothelium-derived factors. The device has two independent flow channels with each channel separated by a three-dimensional region filled with a hydrogel scaffold. C, SMC were cultured in the three-dimensional μFD in co-culture with EC. A type I collagen scaffold was injected into the three-dimensional region, and cells were introduced into their respective channels (smooth muscle channel and endothelial channel). Factors generated by EC were transported across the scaffold by passive diffusion. The image is shown at 4× magnification and rotated 90° relative to schematics shown in A and B. The cumulative effect of KLF2-dependent EC factors act to inhibit EC-directed three-dimensional SMC migration. Shown are 10× magnification fluorescent images of no endothelial control (NoEC) (D), endothelial cells expressing GFP only (EC-GFP) (E), and endothelial cells expressing KLF2 (EC-KLF2) (F). Blue, nuclei (DAPI); red, SMC (PKH-26 membrane stain). G, histogram quantification of the SMC migration distance into the three-dimensional region for the three culture conditions revealed that EC co-culture increases SMC migration compared with no endothelial control and that this effect is abolished by KLF2 induction in EC, as shown by quantifying the total number of SMC invading the three-dimensional area after 48 h (G, inset). Data are reported as the mean of 10 (EC-GFP and EC-KLF2) and nine (NoEC) independent experiments, with error bars representing S.E.* p < 0.05 across all conditions; †, p < 0.05 pairwise comparison between NoEC and EC-GFP only.

The effect of EC KLF2 expression on SMC migration was characterized by co-culturing SMC with KLF2 adenovirus-infected EC in opposite channels of the three-dimensional μFD. SMC migration and invasion into the three-dimensional region was compared between three co-culture conditions: no endothelium (NoEC; Fig. 3D), GFP control adenovirus (EC-GFP; Fig. 3E), and KLF2-GFP adenovirus (EC-KLF2; Fig. 3F). Quantification of three-dimensional SMC migration for the three co-culture conditions revealed that a significantly greater number of SMC invaded the three-dimensional region during co-culture with EC-GFP compared with both the no endothelium and EC-KLF2 co-culture conditions, reported as a histogram distribution of cell number versus migration distance (Fig. 3G), as well as the total number of SMC migrating into the three-dimensional space (Fig. 3G, inset). There was no significant difference when comparing SMC migration in co-culture with noninfected EC to control GFP-expressing EC (data not shown). These three-dimensional SMC migration results demonstrate that the integrative effect of KLF2 expression in EC acts to reduce SMC migration and provide insight into the molecular program used by endothelial cells to control SMC migration.

We next sought to characterize a KLF2-dependent endothelium-derived factor that may account for the observed modulation in three-dimensional SMC migration and focused on CTGF, since it is an EC gene that was found to be down-regulated by the ACC waveform (Fig. 2B). Additionally, CTGF has been shown by others to stimulate migration in multiple cell types (42, 43) and exist at high levels in atherosclerotic lesions, a focal site of vascular disease associated with increased smooth muscle cell migration (44). First, we documented that adenovirus-mediated KLF2 expression in EC (EC-KLF2) reduces endogenous CTGF expression compared with control adenovirus-infected EC (EC-GFP) (Fig. 4A). Consistent with this observation, we also found CTGF promoter activity to be reduced in EC-KLF2 compared with EC-GFP as well as noninfected EC (EC-Only) (Fig. 4B). This result was achieved by employing a luciferase-based activity assay using ~2.0 kb of the CTGF proximal promoter (30), which contains several putative KLF binding sites. Finally, using a gel shift assay and probing for the five 25-bp segments of the 2.0-kb CTGF proximal promoter containing canonical CAATCC KLF binding sites, we demonstrated that KLF2 binds only to the CTGF proximal promoter sequence between base pairs −1730 and −1706 (Fig. 4C).

Once establishing that KLF2 acts to inhibit CTGF transcription and gene expression, the effect of exogenous CTGF on three-dimensional SMC migration was evaluated. 10 μg/ml CTGF was first administered in the absence of EC to the channel opposite of SMC with the three-dimensional μFD migration assay. Quantification of the resulting three-dimensional SMC migration revealed that CTGF significantly increases the total number of SMC migrating into the three-dimensional region after 48 h compared with control (Fig. 4, D and E). Moreover, delivering CTGF at the same concentration to the endothelial cell channel in the presence of EC expressing KLF2 (EC-KLF2) was able to restore three-dimensional SMC migration levels to that of SMC co-cultured with control EC (EC-GFP).

mensional hydrogel and communicate via paracrine signals transported across the hydrogel region (41).
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**FIGURE 4.** *CTGF* expression is reduced by KLF2 and induces SMC migration. Adenovirus-mediated endothelial KLF2 expression (EC-KLF2) reduces endogenous *CTGF* gene expression compared with control adenovirus-infected EC (EC-GFP), as determined by real time TaqMan PCR (A) and CTGF transcriptional activity compared with EC-GFP and negative control EC (EC-Only), as determined by a luciferase activity assay using an ~2.0-kb fragment of the CTGF proximal promoter (B). The binding of KLF2 to the CTGF promoter was documented using a gel shift assay, with GST-KLF2 binding observed in only one (~1730 to ~1706) of the five (25-bp) probes tested from the CTGF proximal promoter (C). All of the probes contained either one or two canonical CACCC KLF binding sites. D, 10× magnification fluorescent images of control (right) and 10 μg/ml CTGF (left) three-dimensional SMC migration. Blue, nuclei (DAPI); red, SMC (PKH-26 membrane stain). E, quantification of the total number of SMC invading the three-dimensional region after 48 h showed that CTGF induced more SMC migration compared with control. F, the addition of 10 μg/ml CTGF to the EC channel of the EC-KLF2 co-culture with SMC restored SMC migration to EC-GFP levels, as shown by normalizing the number of SMC migrating in each test condition to the number migrating in the EC-GFP co-culture condition. Data are reported as the mean of three (A), four (E), or five (B and F) experiments with error bars representing S.E.; *p < 0.05. NS, not significant.

(4F). These results show that KLF2 regulates *CTGF* expression at the transcriptional level and indicate that CTGF is a KLF2-dependent EC factor capable of influencing SMC migration. Taken together, these three-dimensional SMC migration findings suggest that the gene expression changes evoked by collateral flow during coronary collateralization (e.g. ACC waveform) act to limit SMC migration, which may have important implications for the vessel wall structure during flow-mediated adaptive remodeling.

**DISCUSSION**

Environmental cues, circulating humoral factors, and locally produced signals all influence the function and structure of a remodeling vessel (45). In this study, we combined numerical simulation with molecular and functional vascular cell phenotyping analysis and were able to determine that collateral flow controls endothelial gene expression patterns relevant to adaptive remodeling and that the resulting endothelial phenotypic changes have the capacity to modulate both smooth muscle gene expression and function.

Several of the genes characterized by genome-wide transcriptional profiling to be differentially regulated by the coronary collateral waveforms have previously been reported to regulate critical processes involved with adaptive remodeling. Specifically, the ACC waveform increased the expression of genes important for vessel tone (ENOS and VIP), vascular development/remodeling (TIE2, FGF2, DLL1, and JAG1), and maintenance of the arterial extracellular matrix (NOV and TIMP3). Vasodilation of collateral vessels has been observed to be dependent on endothelial nitric-oxide synthase during the initial stage of collateral adaptation (46), whereas in vivo evidence supports a role for the angiopoietin-Tie2 and Notch-Jag1/Dll1 receptor-ligand systems in both arterial development and adaptive coronary remodeling (47–49). The ACC waveform also down-regulated multiple genes involved with angiogenesis/wound healing (*ANGPT2, FBLN5, IL8, CTGF, CYR61, SEMA3A, TGFβ2*, and NRP1), inflammation (IL8 and MCP-1), and vasoconstriction (*END-1*) compared with the NCC waveform. Angiogenic and inflammatory genes are often associated with vascular remodeling, particularly in ischemic tissue or during pathological remodeling, but these processes may have detrimental effects to adaptive remodeling, since their stimuli and end-product vessel structure and function differ significantly. Consistent with the concept that EC genes down-regulated by ACC flow have detrimental effects on adaptive remodeling is the observation that constitutive overexpression of *ANGPT2*, a proangiogenic endothelial gene, in animals subjected to hind limb femoral artery ligation dramatically impairs collateral vessel remodeling (50).

Based on the differential expression of endothelial genes documented here, collateral flow has the ability to evoke distinct endothelial gene expression patterns whose functional contribution toward adaptive arteriogenesis may be through the stabilization and maintenance of large diameter arteries. Furthermore, when we delivered flow-mediated EC conditioned medium to cultured SMC, the SMC displayed gene expression patterns consistent with the assertion that collateral flow promotes vascular wall stabilization. In particular, we observed decreased expression of *MMP-3* and increased expression of *SMC MYOCD* as well as the myocardin transcriptional targets *CNN1* and *αSMA*. The latter observation indicates that collateral flow acting on the endothelium results in paracrine modulation of the contractile...
SMC phenotype, an important event associated with adaptive remodeling.

The transcription factor KLF2 emerged from the collateral flow-mediated EC transcriptional profile analysis as a potential key player in regulating an adaptive remodeling endothelial response based on evidence of its flow dependence and necessity to the function of the developing vessel wall. A member of the Kruppel family of zinc finger transcription factors, KLF2, has been shown to be tightly regulated by flow (31, 51–53) and critical for controlling multiple endothelial phenotypes (31, 51, 54, 55). A number of other endothelial transcription factors were also regulated by the ACC waveform (supplemental material), but KLF2 had the strongest in vivo evidence indicating that the transcription factor may have a critical role in adaptive remodeling, since mice lacking KLF2 display lethal phenotypes of impaired vascular maturation and function. Both KLF2-/- mice and endothelium-specific knock-out of KLF2 in mice result in embryonic lethality, due either to hemorrhage (7, 11) or defects in vascular hemodynamics (8), respectively. Moreover, a recent report by Wu et al. (10) documented that the medial layer of vessels of KLF2-/- mice is poorly developed and that SMC of these mice display a patchy morphology with condensed nuclei when compared with wild-type mice. None of these mouse models, however, displayed impaired vasculogenic capacity, implying that the principal vascular defect associated with KLF2 loss involves ineffective regulation, potentially via the endothelium, of smooth muscle functions associated with vessel wall stability and reactivity.

This evidence prompted us to investigate the functional effect of EC KLF2 expression on interactions with SMC important for adaptive remodeling. Using three-dimensional SMC migration as a functional SMC read-out, our results show that EC KLF2 expression suppresses SMC migration and that this effect can be rescued by the exogenous delivery of CTGF, an EC-secreted factor whose expression is down-regulated by KLF2 at the transcriptional level. Furthermore, using a gel shift assay, we demonstrated that KLF2 can directly bind the CTGF proximal promoter sequence located at −1730 to −1706. This region of the 2-kb CTGF proximal promoter was one of two probes tested that contains two canonical CACC KLF binding sites in tandem, whereas the other three probes contain a single CACCC site. Taken together, these data support the hypothesis that KLF2 has a role in adaptive remodeling by controlling EC-dependent SMC function and suggest that improper regulation of SMC migration may contribute to the vascular wall defects observed in KLF2 knock-out mice. Furthermore, the three-dimensional μFD migration assay as used in these experiments does not allow for cell contact between EC and SMC, indicating that the effect of EC KLF2 expression on SMC migration is partially due to the differential regulation and release of CTGF and other yet to be identified EC factors.

Overall, the results documented here demonstrate that collateral flow regulates cellular events critical for adaptive remodeling, including endothelial gene expression and endothelium-derived paracrine signals that modulate smooth muscle gene expression and functional phenotypes. In particular, endothelial genes regulated by collateral flow were found to control smooth muscle migration, a finding that has important implications for understanding how biomechanical forces influence endothelial interactions with smooth muscle cells. Finally, these data indicate that an integrated endothelial response to flow during adaptive remodeling controls critical events relevant to the blood vessel wall architecture.

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