The miR-143/145 Cluster Is a Novel Transcriptional Target of Jagged-1/Notch Signaling in Vascular Smooth Muscle Cells

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Activation of Notch signaling by Jagged-1 (Jag-1) in vascular smooth muscle cells (VSMC) promotes a differentiated phenotype characterized by increased expression of contractile proteins. Recent studies show that microRNAs (miR)-143/145 regulates VSMC phenotype. The serum response factor (SRF)/myocardin complex binds to CaRg sequences to activate miR-143/145 transcription, but no other regulators are known in VSMC. Using miR arrays, we found miR-143/145 induced following expression of a constitutively active Notch1 intracellular domain (N1ICD). We hypothesized that miR-143/145 is required for Jag-1/Notch-induced VSMC differentiation. Activation of Notch receptors by Jag-1 caused CBF1-dependent up-regulation of miR-143/145, increased differentiation, and decreased proliferation. Conversely, inhibiting basal Notch signal decreased steady state levels of miR-143/145. Using SRF knockdown, we found that Jag-1/Notch induction of miR-143/145 is SRF independent, although full acquisition of contractile markers requires SRF. Using miR-143/145 promoter reporter constructs we show Jag-1/Notch increases promoter activity, and this is dependent on intact CBF1 consensus sites within the promoter. Chromatin immunoprecipitation (ChIP) assays revealed that N1ICD-containing complexes bind to CBF1 sites in the miR-143/145 promoter. We also identified N1ICD complex binding to CBF1 sites within the endogenous human miR-143/145 promoter. Using miR-143/145-interfering oligonucleotides, we demonstrate that Jag-1/Notch signaling requires induction of both miR-143 and miR-145 to promote the VSMC contractile phenotype. Thus, miR-143/145 is a novel transcriptional target of Jag-1/Notch signaling in VSMC. We propose miR-143/145 as activated independently by Jag-1/Notch and SRF in parallel pathways. Multiple pathways converging on miR-143/145 provides potential for fine-tuning or amplification of VSMC differentiation signals.

Vascular smooth muscle cell (VSMC) plasticity is required during postnatal vascular development and vascular remodeling in response to injury (1). In quiescent vasculature, VSMC reside in the tunica media of the blood vessel wall where they provide structural support and contractile forces necessary for homeostasis (2). VSMC respond to vascular injury with decreased expression of contractile proteins and increased proliferation and migration (3). Several pathways have been identified that promote the VSMC contractile phenotype including SRF/myocardin (4), transforming growth factor β (5, 6), and Notch signaling (7, 8), while transcription factors such as Krüppel-like factor 5 (KLF5) are known to promote a proliferative phenotype by direct binding to the cyclin D1 promoter (9).

Aberrant Notch signaling in the vasculature is associated with vascular defects including abnormal structure, leakiness, Alagille syndrome, and CADASIL (10, 11). Normally, VSMC express Notch1 and Notch3, endothelial cells Notch1 and Notch4, and both cells types express Jagged-1 (Jag-1) and Delta-like-1 (Dll-1) in a context dependent manner (12). Activation of Notch receptors by their transmembrane ligands leads to cleavage of the intracellular domain (ICD) of the receptor via the γ-secretase complex (13). NotchICD translocates to the nucleus, complexes with the transcriptional regulator CBF1 (14), and activates downstream transcriptional targets including HES and HRT. In the vasculature, expression of Jag-1 by the endothelium promotes VSMC differentiation by activating Notch3 (15), while the physiological functions of Dll-1/Notch signaling in VSMC are not known. Although Jag-1 is critical for VSMC differentiation in vivo (16), specific downstream mechanisms are not well characterized.

MicroRNAs (miR) are critical regulators of cardiovascular development, homeostasis, and repair of injury (17–19). The human miR-143/miR-145 (miR-143/145) cluster on chromosome 5q33 is regulated by a common promoter (20). miR-143/145 are transcriptional targets of SRF via the CaRg box sequence, and act to promote VSMC differentiation through translational repression of proliferation associated proteins KLF5 (21) and member of the ETS oncogene family (Etk1) (22). Targeted deletion of the mouse miR-143/145 cluster showed it to be dispensable for VSMC differentiation, although it is required for the maintenance of VSMC cytoskeletal architec-

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The abbreviations used are: VSMC, vascular smooth muscle cell; Jag-1, Jagged-1; N1ICD, Notch1 intracellular domain; SRF, serum response factor; KLF, Krüppel-like factor; miR, microRNA; ChIP, chromatin immunoprecipitation; GSI, γ-secretase inhibitor; SM-actin, α-smooth muscle actin.

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ture and thus, regulation of blood pressure (23). In addition, VSMC response to vascular injury is affected in null miR-143/145 mice (24, 25), similar to loss of function phenotypes of Notch1 (26).

In this report, we identify miR-143/145 as a novel, CBF1-dependent target of Jag-1/Notch signaling, and define its role in VSMC differentiation. Additionally, we show regulation of miR-143/145 by Jag-1/Notch signaling is at the transcriptional level, and independent of SRF/myocardin activity and the CArG sequence in the miR-143/145 promoter/enhancer regions. Instead, consensus CBF1 sites in the cardiovascular enhancer are responsive to Jag-1/Notch signaling. Knockdown of miR-143/145 in vitro revealed that acquisition of the contractile phenotype by Jag-1/Notch signaling requires both miR-143 and miR-145. Thus, our findings reveal an alternate pathway regulating miR-143/145 expression and have implications for vascular diseases that involve modulation of VSMC contractile activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All experiments were conducted using primary human aortic VSMC (Lonza, Walkersville, MD) cultured in smooth muscle growth media (SmGM) (Lonza) between passages 5–7.

**Ligand Activation of Notch Receptors**—Immobilization of control Fc, or Jag-1 Fc was performed as described (27). Briefly, 2 μg Fc-specific human IgG (Sigma) dissolved in PBS was added to each well of a 12-well plate and incubated for 2 h at room temperature. The solution was then aspirated and 1 μg of either purified Fc or recombinant rat Jag-1 Fc chimera (R&D Systems, Minneapolis, MN) was resuspended in PBS, added to each well, and incubated at 4 °C overnight. The next morning, the solution was aspirated before plating cells.

**Adenoviral Transduction**—VSMC were treated with 3000 viral particles (vp)/cell for 6 h in smooth muscle basal media (SmBM) with Gene-juice (Invitrogen, Carlsbad, CA). After 6 h, virus was removed, cells washed 3× with PBS, and incubated in SmGM.

**miR Array**—Passage 5 human aortic VSMC were transduced with 3000 vp/cell of Notch1ICD or control GFP adenovirus (28, 29) in triplicate for 6 h in SmBM. Cells were washed in PBS and allowed to grow for 24 h in SmGM. Total RNA was collected in Tri Reagent (Sigma) and processed for miR array analysis (Exiqon miRCURY microRNA array version 11.0, Denmark).

**miR Analysis and qRT-PCR**—For miR quantitative RT-PCR, RNA was isolated using miRCURY RNA isolation kit for cell and plant (Exiqon), and reverse transcribed using miRCURY First Strand Synthesis Kit (Exiqon). miR sequences were amplified using LNA primers (Exiqon) and SyBR green supermix (Bio-Rad). Cycling conditions were 95 °C for 10 min, 40 cycles at 95 °C for 10s and 60 °C for 1 min with a cooling ramp rate of 1.6 °C/s. The data were normalized to 5S rRNA as an internal control and immobilized Fc as a biological control. For quantitative RT-PCR, total RNA was isolated using Tri Reagent and reverse transcribed using qScript (Quanta Bioscience, Gaithersburg, MD). Amplification was carried out with SyBR green supermix using a primer annealing temperature of 58 °C for 40 cycles. The data were normalized to β-actin amplification followed by the respective biological control. Primers were generated using Primer 3 (30).

**Western Blot**—Western blots were performed as described (29). Primary antibodies for α-smooth muscle actin (SM-actin), calponin1 (calponin), β-tubulin (tubulin) (Sigma), SM22α (Abcam, Cambridge, MA), V5 (Invitrogen), GAPDH (Cell Signaling, Danvers, MA) and SRF (Santa Cruz, Santa Cruz, CA) were diluted 1:1000 and incubated at 4 °C overnight. Secondary HRP-conjugated anti-mouse/rabbit antibodies (Sigma) were diluted 1:6000 and incubated for 1 h at room temperature followed by detection using ECL (Pierce). Western blot data were quantified using ImageJ software based on integrated pixel density of bands in experimental and control groups normalized to β-tubulin or GAPDH.

**SRF/miR-143/145 Knockdown**—Transfections were performed using the human aortic smooth muscle cells optimized nucleoefector kit and electroporator (Amaza). Knockdown of SRF was accomplished using 125 pmol siRNA or scrambled All Star Control (Qiagen, Valencia, CA) with 5 × 10^5 cells and electroporated using program U-025. Cells were then cultured for 48 h before plating on immobilized Jag-1 Fc or control Fc. Knockdown of miR-143 or miR-145 was performed as described above using 2 pmols of miR-143 or miR-145 power inhibitors or a non-targeting control (Exiqon) before plating on immobilized Jag-1 Fc or Fc control for designated time points.

**Luciferase Assays**—For KLF5 3′ UTR luciferase assays, 1 × 10^6 VSMC were transfected with 5 μg of pGL3-KLF5 3′ UTR vector (21), 2 μg of Renilla and 2 μg of N1ICD or GFP plasmid using the V-025 program (Amaza nucleoefector–Lonza) and allowed to grow for 48 h before analysis of luciferase activity. For KLF5 3′-UTR rescue experiments, 5 μg of pGL3-KLF5 3′ UTR vector and 2 μg of Renilla were co-transfected with either nRNA or si-miR-145 oligonucleotides before plating on Jag-1 Fc or Fc control for 24 or 48 h.

**miR-143/145 β-Galactosidase Reporter Assays**—We utilized previously characterized miR-143/145 LacZ reporter constructs (23) that were generously provided by Dr. Eric Olson from The University of Texas Southwestern Medical Center (Dallas, Texas). Reporter A contains the region 0 to −5 kb upstream of the miR-143/145 transcriptional start site, and Reporter B contains the −3 kb to −4.6 kb cardiovascular enhancer upstream of Hsp68-LacZ. These constructs with either a wild type or mutated CArG box were utilized. VSMC at passage 5 were co-transfected with 2 μg of pGL3 (luciferase vector) and 3 μg of either wild type or CArG box mutant reporter A or reporter B before being immediately plated on Jag-1 or control Fc for 24 h. β-Galactosidase activity was quantified using Novabright β-galactosidase and Firefly Luciferase Dual Enzyme Reporter Gene Chemiluminescent Detection System (Invitrogen). β-Galactosidase activity was normalized to luciferase as an internal control and to Fc plated cells as a treatment control.

**Chromatin Immunoprecipitation Assays (ChIP)**—2 × 10^6 VSMC were adenovirally transduced with LacZ or V5-tagged N1ICD (3000 vp/cell) for 6 h. The virus was removed, and cells were allowed to grow for 48 h before being collected for ChIP analysis (Millipore, Billerica, MA). Cells were fixed in formaldehyde for 10 min, lysed, and sonicated (29). Lysates from LacZ
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Transduced cells were incubated with mouse monoclonal anti-V5 antibody at 1:1000 for a biological control, while N1ICD transduced cell lysates were incubated with a human IgG antibody (Sigma) at 1:1000 for an antibody control or anti-V5 for an experimental group. The crosslinks on immunoprecipitated complexes were reversed using 5 M NaCl and heat, DNA isolated and purified by phenol/chloroform extraction and subjected to PCR using human primers flanking predicted CBF1 binding sites within the miR-143/145 promoter. Cycling conditions were 95 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. PCR products were analyzed on a 2% agarose gel. For Reporter B ChIP assays, 2 × 10⁶ VSMC at P5 were transfected with 5 μg of CArG box mutant reporter B and allowed to recover for 24 h before being transduced with 3000 vp/cell N1ICD for 6 h in serum free media. After removal of the virus, cells were allowed to recover for 48 h. The ChIP assay was performed as above using anti-V5 antibody at 1:1000 for the immunoprecipitating antibody, and human IgG antibody (1:1000) as a specificity control.

Site-directed Mutagenesis—Point mutations in CBF1 consensus binding sites within the CArG mutant reporter B were made according to Invitrogen’s Gene Tailored site-directed mutagenesis kit (now called GeneART mutagenesis system). Briefly, the mutagenesis reactions were performed using Platinum TaqDNA polymerase (Invitrogen), with specifically designed mutagenesis primers and cycling conditions as follows: 95 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 61 °C for 30 s and 68 °C for 10 s. Each mutagenesis product was transfected into chemically competent DH5α TIR Escherichia coli (Invitrogen) and grown at 37 °C overnight. Colonies were selected, and screened for the TGGGAA to TAAGAA mutation at each site by DNA sequencing.

Immunostaining—For smooth muscle contractile proteins and tubulin, passage 5 human VSMC were plated on immobi- lized Fc or Jag-1 Fc for 48 h. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and incubated with primary antibodies (1:200) for 1 h. Cells were then incubated with goat anti-mouse Alexa-fluor-conjugated secondary antibodies (Invitrogen) at 1:200 for 1 h. For actin staining, cells were first stained for tubulin, then incubated with Texas Red-conjugated phalloidin diluted 1:100 in PBS for 15 min at room temperature. DAPI staining was done using 1 μg/ml DAPI in PBS for 5 min. For BrdU incorporation, cells were plated on Jag-1 Fc for 42 h prior to being pulsed with 10 μM BrdU for 6 h. Cells were then fixed in 4% paraformaldehyde, washed in 4N HCl for 30 min and stained using Alexa-Fluor 488 conjugated mouse anti-BrdU monoclonal antibody (Millipore) diluted to 1:200 for 1 h at room temperature. The percentage of BrdU-positive cells was calculated from n = 15 individual pictures/treatment.

Statistical Analysis—All statistical analysis was performed using Student’s t test with a significance cutoff of p < 0.05. Data are presented as mean values ± S.E.

RESULTS

The miR-143/145 Cluster Is Differentially Regulated by Notch Signaling in Human VSMC—We previously reported that activation of Notch signaling by expression of the N1ICD enhanced the contractile phenotype in human primary VSMC (29). We used this system to screen for miR regulated after Notch activation. Thirteen miR were significantly regulated (Fig. 1A). Among these, miR-145 was the highest significantly up-regulated gene selected for further validation and study. miR-143, which is clustered with miR-145, was also induced in the array. Using quantitative PCR, we analyzed expression of miR-143/145 and validated a ~7 fold increase in miR-143 and a ~15 fold increase in miR-145 following N1ICD expression in VSMC (Fig. 1B). This Notch-dependent induction was inhibited using dominant negative (dn) CBF1, suggesting that the signaling is through the canonical CBF1-dependent Notch pathway.

To test regulation of miR-143/145 following ligand activation of endogenous Notch receptors, we utilized an immobilized recombinant Jag-1 Fc chimera. Human aortic VSMC express Notch1 and Notch3 receptors (5), and we have observed strong expression of Notch2 as well (data not shown). Similar to the activity of N1ICD, Jag-1 activation of Notch receptors resulted in significant up-regulation of miR-143/145 (Fig. 1C). The induction of miR-143/145 expression by Jag-1 was abolished by inclusion of 0.5 μg/ml γ-secretase inhibitor (GSI) to the cultures, which blocks endogenous Notch proteolysis and thus activation. We next tested the role of CBF1, which is activated in VSMC following stimulation by Jag-1 (data not shown). Expression of dnCBF1 in VSMC significantly reduced the ability of Jag-1 to induce miR-143/145 expression (Fig. 1D), implying that the effects on miR-143/145 expression by Jag-1 are mediated by canonical CBF1-mediated Notch signaling. To determine the role of Notch signaling in regulating basal miR-143/145 levels, we plated VSMC in the presence of increasing concentrations of GSI for 48 h and analyzed the effects on miR-143/145 expression levels as well as VSMC contractile proteins. Incubation of VSMC with 0.5 μg/ml GSI results in a ~40-fold and ~30-fold decrease in miR-143 and miR-145 levels, respectively (Fig. 1E). Suppression of miR-143/145 transcripts by GSI was dose dependent. Consistent with a role for Notch signaling in the basal VSMC contractile phenotype, VSMC incubated in the presence of 0.5 μg/ml GSI for 48 h displayed reduced SM-actin, calponin and SM22α transcript (Fig. 1F) and protein (Fig. 1G). These data suggest that basal endogenous Notch signaling is involved in the regulation of miR-143/145 expression, the contractile machinery, and VSMC phenotype.

Jag-1/Notch Signaling Promotes VSMC Differentiation at the Expense of Cell Proliferation—The miR-143/145 cluster has been shown to be a critical mediator of VSMC differentiation by repressing cell proliferation (22). We asked whether Jag-1/Notch signaling promotes a contractile phenotype at the expense of cell proliferation. Jag-1 activation of Notch receptors significantly up-regulates SM-actin and SM22α transcript levels (Fig. 2A) by 30h. Increased expression of contractile proteins SM-actin, calponin and SM22α was also observed by 48 h (Fig. 2B). We observed a notable difference in VSMC morphology consistent with an increased contractile phenotype when plated on Jag-1. Using immunofluorescence, we show robust increases in SM-actin and calponin levels in VSMC plated on Jag-1 but observed no differences in the arrangement of the F-actin/microtubule cytoskeleton when compared with Fc at 48 h (Fig. 2C). Thus, changes in morphology in response to
Jag-1/Notch signaling are likely due to increased contractile proteins within the cell and not a rearrangement of the cellular cytoskeleton. To better understand if Jag-1/Notch signaling affects VSMC proliferation, we quantified total cell number per field in Fc- and Jag-1-plated cultures and observed that Jag-1 resulted in a significant reduction in total cell number after 48 h (Fig. 2D). To ensure that reduced cell number was due to decreased proliferation, we plated VSMC on Fc or Jag-1 for 42 h before pulsing with a final concentration 10^{-6} M 5-bromo-2'-deoxyuridine (BrdU) for 6 h. Jag-1 stimulation led to a significant reduction in the percentage of BrdU-positive nuclei compared with Fc (Fig. 2E). Collectively, our data show that Jag-1/Notch signaling promotes a contractile phenotype at the expense of proliferation in VSMC. These findings are consistent with increased miR-143/145 levels observed by Jag-1/Notch signaling.

Jag-1/Notch Signaling Regulates miR-143/145 Independent of SRF—Previous reports identified a region in the miR-143/145 promoter that contains a highly conserved SRF binding site (CArG box) for SRF/myocardin-dependent transcription (23). We performed a series of experiments to determine if regulation of miR-143/145 is dependent on the SRF/myocardin pathway; and asked whether Notch signaling regulates SRF/myocardin levels in the cell as an indirect means of increasing miR-143/145 expression. Activation of Notch receptors with Jag-1 or expression of N1ICD in VSMC increased SM-actin by 48 h without any observable change in SRF protein levels (Fig. 3A). Additionally, no significant changes in myocardin (a potent co-

| Human microRNA | Mean GFP | N1ICD | ΔLMR | Fold Change N1ICD vs. GFP | T-Test N1ICD vs. GFP |
|----------------|---------|-------|------|---------------------------|---------------------|
| miR-145        | -1.58   | 0.16  | 1.74 | 3.33                      | 5.98E-03            |
| miR-143        | -1.51   | -0.04 | 1.47 | 2.78                      | 9.42E-02            |
| miR-1184       | -1.09   | -0.25 | 0.83 | 1.78                      | 1.68E-02            |
| miRPlus-E1045  | -0.91   | -0.22 | 0.69 | 1.62                      | 4.60E-02            |
| miRPlus-D1036  | -0.90   | -0.21 | 0.69 | 1.62                      | 2.55E-02            |
| miRPlus-F1195  | -0.88   | -0.22 | 0.66 | 1.58                      | 3.39E-02            |
| miR-1908       | -0.98   | -0.32 | 0.65 | 1.57                      | 4.54E-02            |
| miRPlus-A1098  | -0.81   | -0.20 | 0.60 | 1.52                      | 4.93E-02            |
| miRPlus-F1029  | -0.69   | -0.18 | 0.58 | 1.49                      | 3.47E-02            |
| miR-1275       | -0.53   | -0.16 | 0.37 | 1.29                      | 2.43E-02            |
| miRPlus-F1024  | -0.57   | -0.28 | 0.29 | 1.22                      | 9.12E-03            |
| miRPlus-F147   | -0.41   | -0.80 | -0.39| 0.76                      | 1.73E-02            |
| miR-299-3p     | 0.07    | -0.76 | -0.83| 0.56                      | 3.89E-02            |

FIGURE 1. The miR-143/145 cluster is regulated by Notch signaling in human VSMC. A, miR array analysis of VSMC with activated Notch signaling. Shown are the miR that were significantly regulated following Notch activation. Although miR-143 showed a trend toward increased expression, it was not significantly different in the array (gray shading). B, qPCR analysis of miR-143/145 in response to expression of N1ICD in the presence or absence of dominant negative CBF1 (dnCBF) for 36 h. p < 0.01 for N1ICD induction and dnCBF1+ N1ICD inhibition of *, miR-143 and **, miR-145. C, mi qPCR analysis of miR-143/145 activation at 24 h by Jag-1 Fc in the presence or absence of 0.5 μg/ml GSI, an inhibitor of endogenous Notch signaling, or DMSO control. D, qPCR analysis of miR-143/145 activation at 24h by Jag-1 Fc in the presence or absence of adenovirally transduced dnCBF1, an inhibitor of canonical Notch signaling. E, qPCR analysis of endogenous miR-143/145 levels at 48 h in response to increasing concentrations of GSI. Analysis of SM-actin, calponin, and SM22α at the transcript (F) and protein level (G) after 48 h of incubation with 0.5 μg/ml G3 or DMSO control.
activator of SRF activity) transcripts were observed under these conditions (data not shown). We then tested the requirement of SRF for the Jag-1/Notch induction of miR-143/145 using an SRF-specific small interfering oligonucleotide (siRNA-SRF). This siRNA was effective in reducing SRF protein by >70% at 48–72 h post-transfection (Fig. 3B and supplemental Fig. S1B).

FIGURE 2. Jag-1/Notch signaling promotes a contractile phenotype at the expense of cell proliferation. A, analysis of VSMC contractile markers SM-actin, calponin, and SM22α at the transcript (A) and protein (B) level post-stimulation of Notch receptors with Jag-1 Fc for 30 and 48 h, respectively. C, immunofluorescence analysis of SM-actin, calponin, and the F-actin (phalloidin)/β-tubulin cytoskeleton after 48 h stimulation with Jag-1 Fc or Fc control. Scale bar, 50 μm. D, quantification of cell number/field (n = 10 fields/group) and representative phase contrast images after 48 h stimulation with Jag-1 Fc or Fc control (scale bar, 200 μm). E, quantification of BrdU-positive nuclei (n = 15 fields/group) and representative immunofluorescent images of BrdU incorporation after 48 h of stimulation with Jag-1 Fc or Fc control. VSMC were plated on Jag-1 Fc or Fc control for 18 h before pulsing with a final concentration in culture of 10 μM BrdU for 6 h, and being subjected to immunostaining using anti-BrdU antibody (scale bar, 200 μm).

FIGURE 3. Jag-1/Notch signaling regulates miR-143/145 independent of SRF. A, analysis of SRF and SM-actin levels in response to Jag-1 activation of Notch receptors (left) or expression of Notch1ICD (cells transduced with 3000 vp/cell for 6 h followed by 48 h of incubation). B, VSMC were transfected with 125 pmol of a non-targeting control oligonucleotide (ntRNA) or a small interfering oligonucleotide specifically targeting SRF (siRNA-SRF). SRF protein levels in control and knockdown cells were analyzed 48 h post-transfection. C, qPCR analysis measuring the relative induction of miR-143/145 by Jag-1/Notch at signaling as compared with Fc control at 24 h in SRF knockdown VSMC. Prior to plating on Jag-1 Fc or Fc control, VSMC were transfected with 125 pmol of siRNA-SRF or ntRNA and allowed to recover for 48 h. D, SM-actin transcript levels in control and SRF knockdown VSMC stimulated with Jag-1 Fc or Fc control for 48 h. E, SM-actin protein level was analyzed by immunoblot (left) and quantified (right) in control and SRF knockdown VSMC stimulated with Jag-1 Fc or Fc control for 48 h. F, SM-actin transcript levels in control and SRF knockdown VSMC constitutively expressing N1ICD. G, immunoblot analysis of SRF and SM-actin protein (left) and respective quantification of SM-actin levels (right) in control or SRF knockdown VSMC constitutively expressing GFP or N1ICD for 48 h.
As expected, knockdown of SRF led to dramatic suppression of the SRF transcriptional targets miR-143/145 and SM-actin (supplemental Fig. S1, C and D). To determine if Jag-1/Notch signaling can induce miR-143/145 independent of SRF activity, VSMC transfected with 125 pmol of non-targeting control (ntRNA) or siRNA-SRF were grown for 48 h, plated on Jag-1 or Fc for 24 h, and subjected to qPCR analysis for miR-143/145 expression. Although the basal levels of miR-143/145 were considerably lower in siRNA-SRF-treated cells, the relative induction by Jag-1/Notch was unchanged from that of control ntRNA cells (Fig. 3C). Thus, Jag-1/Notch maintained full ability to induce miR-143/145 transcript when SRF levels were suppressed.

Because regulation of miR-143/145 by Jag-1/Notch signaling is SRF independent, we tested if Notch signaling could still promote a contractile phenotype in SRF knockdown VSMC. Cells receiving 125 pmol of ntRNA and plated on Jag-1 for 48 h showed increased SM-actin transcript and protein levels when compared with Fc. In contrast, cells receiving siRNA-SRF showed a significant reduction in the ability of Jag-1/Notch to increase SM-actin transcript (Fig. 3D) and protein levels (Fig. 3E). To challenge the system, these same investigations were carried out using N1ICD, which provides a more robust activation of the pathway, leading to a stronger differentiation signal. Expression of N1ICD in cells transfected with ntrRNA caused a dramatic increase in SM-actin transcript and protein levels when compared with a GFP control, while cells receiving 125 pmol of non-targeting control siRNA-SRF and expressing N1ICD displayed significant reduction of the pathway, leading to a stronger differentiation signal. These data indicate that regulation of miR-143/145 by Notch signaling is independent of SRF; however, SRF is required for full acquisition of the contractile phenotype by Notch signaling.

The miR-143/145 Cluster Is a Transcriptional Target of Notch Signaling in VSMC—To determine whether Jag-1/Notch regulation of miR-143/145 is at the transcriptional level, we utilized two previously characterized miR-143/145 reporter constructs (23). Reporter A contains the region 0 to −5.5 kb upstream of the miR-143/145 transcriptional start site and harbors a wild type (WT) or mutated (MUT) SRF binding site [CC(A/T)nGG] (CARG box). Reporter B contains the region from −3.0 kb to −4.6 kb upstream of the miR-143/145 start site, which was defined as a cardiovascular enhancer sufficient to regulate VSMC expression of miR-143/145 in vivo in transgenic mice. We utilized reporters A or B with either a WT or MUT CARG box, which abolishes SRF interaction. To determine if Jag-1/Notch signaling increases miR-143/145 promoter activity, we transfected VSMC with 2 μg of pGL3 vector and 3 μg of either WT or MUT reporter A or reporter B and plated on Jag-1 or Fc in the presence of 0.5 μg/ml GSI or DMSO control for 24 h before assaying for β-galactosidase activity. Jag-1/Notch signaling resulted in a significant increase in WT and MUT reporter A activity (Fig. 4, A and B, respectively). Inhibition of Notch signaling using 0.5 μg/ml GSI abolished this effect. Likewise, Jag-1/Notch signaling significantly increased the activity of WT and MUT reporter B constructs (Fig. 4, C and D, respectively), and this effect was abolished by inclusion of GSI. These data show transcriptional activity of Jag-1/Notch signaling independent of the CARG box sequence in the miR-143/145 promoter.

To further show that Notch signaling directly regulates miR-143/145 via interaction within the cardiovascular enhancer sequence, we designed an experiment to test whether CBFI/NCID complexes are directly bound to MUT reporter B. MUT reporter B contains three CBFI consensus binding sites present within reporter B harboring a mutated SRF binding site (CARG box). Bottom, ChIP assay of N1ICD-complexes bound to reporter B at the specified CBFI binding sites. VSMC were transfected with 5 μg of CARG mutant reporter B allowing to recover for 24 h, and transduced with 3000 vp/cell V5-tagged constitutively active N1ICD for 6 h followed by 48 h of incubation. The control nonspecific anti-human IgG and immunoprecipitating anti-V5 antibodies were incubated with sonicated lysates for 8 h at 4°C before immunoprecipitation, purification, and PCR amplification using primers flanking the indicated CBFI consensus site.

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Notch Transcriptionally Regulates miR-143/14 via CBF1 Binding Sites—We sought to investigate the consequences of specific mutations of each CBF1 consensus site within MUT reporter B (sites 1–3, Fig. 4E). Three separate mutations using MUT reporter B as the starting template were used to create miR-143/145 constructs that have individual mutations in each of the three CBF1 consensus sites located at −3973, −3255, and −3048 with relation to the transcription start site (Fig. 5, A–C). For each mutation, the first two GG were changed to AA. Mutation at these sites has been validated to abolish CBF1 interaction (31). Each CBF1 mutant reporter construct was compared with MUT reporter B (WT CBF1 sites) for miR-143/145 reporter activity at a basal level (endogenous expression) and after 24 h stimulation with Jag-1 Fc. Mutation of the CBF1 binding site at site 1 nearly abolished basal reporter activity, and significantly suppressed induction by Jag-1 Fc (Fig. 5A). By contrast, the site 2 mutation only led to a ~20% decrease in basal reporter activity, and led to a significant increase in reporter activity following Jag-1 Fc stimulation (Fig. 5B). The site 3 mutation had a modest effect on basal reporter activity, similar to the site 2 mutant (Fig. 5C). However, this mutant at site 3 efficiently suppressed the ability of Jag-1 Fc to induce reporter activity (Fig. 5C). These data highlight the requirement of CBF1 site 1 and site 3 for transcriptional activation by Jag-1/Notch signaling.

To determine if CBF1 sites bind to NICD-containing complexes in the endogenous human gene, we performed analysis of the human miR-143/145 promoter and found seven predicted CBF1 consensus sites located within the region 2000 bp upstream from the transcriptional start site. Consensus CBF1 sites were identified within the 0 to −2000 bp region upstream from the human miR-143/145 transcription start site. Using immunoprecipitation with anti-V5, we identified two regions of the endogenous miR-143/145 promoter (sites D and F) that immunoprecipitated with NICD complexes (Fig. 5D). No amplification was observed in NICD-transduced samples immunoprecipitated with normal IgG or LacZ-transduced samples immunoprecipitated with anti-V5.

Jag-1/Notch Signaling Requires miR-143 and miR-145 to Induce a Contractile Phenotype in VSMC—To gain insight into the significance of transcriptional regulation of miR-143/145 by Jag-1/Notch signaling, we obtained miR-143 and miR-145 specific antagomirs (si-miR-143 and si-miR-145, respectively), which decrease their expression. A dose response analysis allowed us to determine that 2 pmol of miR-143 or miR-145 antagomir resulted in a 4–6-fold decrease in miR143/145.
which is comparable to the level of Jag-1/Notch induction of each miR (Fig. 6A). To test the effect of miR-143 and miR-145 suppression, VSMC were transfected with 2 pmol of a non-targeting control (ntRNA), si-miR-143 or si-miR-145 and allowed to recover for 48 h. Cells were then immunostained to detect SM-actin and SM22α. There were no significant changes in the expression of SM-actin or SM22α protein in response to specific knockdown of miR-143 or miR-145 (Fig. 6B). However, concentrations of 10 pmol or higher of si-miR-143 or si-miR-145 suppressed miR143/145 levels by ~10 fold, which resulted in decreased expression of SM-actin and SM22α (data not shown). Because this knockdown experiment was designed to eliminate the Jag-1/Notch induction back to pre-stimulation levels, we used 2 pmol of ntRNA, si-miR-143 or si-miR-145 and plated on Jag-1 or Fc for 48 h, followed by analysis of VSMC markers by immunoblot. Quantification of protein levels revealed that suppression of either miR-143 or miR-145 was able to antagonize the Jag-1-induced increase in SM-actin and SM22α protein, and suppression of miR-143 suppressed Jag-1 induction of calponin (Fig. 6C). To further show the potency of this induction by Jag-1/Notch signaling, we performed the above experiments using half the amount of knockdown probe (1 pmol) and achieved a ~2 fold reduction of miR-143 and ~3-fold reduction of miR-145 (supplemental Fig. S2A). Investigation of SM-actin, calponin, and SM22α in cells transfected with 1 pmol control oligonucleotides or si-miR-143 and plated on Fc control or Jag-1 Fc for 48 h, showed that Jag-1/Notch induction of contractile protein is significantly reduced when miR-143 or miR-145 levels are even partially reduced (supplemental Fig. S2, B and C). These results confirm that Jag-1/Notch promotion of the VSMC contractile phenotype requires miR-143/145.

KLF5 is a documented target of miR-145 via sequences in the 3′-UTR (21). Therefore, we used a KLF5 3′-UTR reporter to analyze miR-145 functional activity. Because of the robust activation of miR-145 by N1ICD, we utilized this method of Notch activation. Expression of N1ICD resulted in a dramatic down-regulation of KLF5 3′-UTR reporter activity compared with the GFP control (Fig. 6D). Using a more robust knockdown con-
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**FIGURE 7. Model of parallel miR-143/145 regulatory pathways in VSMC.** Activation of Notch receptors by Jag-1 leads to proteolytic cleavage of the NICD, translocation to the nucleus, and complex formation with CBF1 to transcriptionally activate miR-143/145. Jag-1/Notch-mediated activation is CBF1-dependent, but SRF-independent. Notch-responsive CBF1 sites flank the CArG box within the previously defined cardiovascular enhancer (23). We propose that while the Notch and SRF pathways independently activate miR-143/145, potential interaction of these pathways may occur to increase miR-143/145 expression. miR-143/145 is required for the reduced cell proliferation and increased acquisition of contractile phenotype by Jag-1/Notch signaling. VSMC differentiation is further promoted by Notch and SRF/myocardin via direct transcriptional activation of VSMC contractile genes by both pathways.

**DISCUSSION**

This study identifies a novel pathway in which Jag-1/Notch signaling induces miR-143/145, which is required for promoting VSMC differentiation. Our data support a model (Fig. 7) in which the miR-143/145 is transcriptionally regulated by canonical Jag-1/Notch signaling in a pathway that is independent and parallel to the SRF pathway. Sequences important for the Jag-1/Notch mediated induction of miR-143/145 reside in the previously defined cardiovascular enhancer sequence, flanking the SRF-responsive CArG box. Therefore, we propose that the contractile phenotype and miR-143/145 expression in human VSMC is promoted by basal levels of endogenous Notch and SRF signaling. *In vitro* propagation of human VSMC favors a proliferative phenotype with moderate levels of VSMC marker expression. However, Notch activation by Jag-1, which is a potent differentiation signal (32), leads to transcriptional activation of miR-143/145 by interaction of NICD-containing complexes at specific CBF1 consensus sites, suppression of proliferation, and up-regulation of VSMC contractile gene expression. Mutation of any of the three CBF1 consensus sites within the miR-143/145 cardiovascular enhancer reduced endogenous reporter activity. However only mutation in CBF1 sites located at −3973 and −3048 reduced Jag-1/Notch activation of reporter activity. The CBF1 consensus site at −3255 may play a role providing negative feedback in response to Jag-1/Notch signaling, thereby fine tuning regulation of the promoter. Our miR-143/145 knockdown studies suggest that minor perturbations in the induction of either miR-143 or miR-145 by Jag-1/Notch signaling are adequate in significantly reducing the induction of contractile proteins, and thus place great importance on miR-143/145 as a novel gene target of Notch signaling in VSMC. Shifting to a contractile phenotype corresponded to a decrease in VSMC proliferation as indicated by decreased BrdU staining. In addition, Jag-1/Notch activation regulates KLF5, a known miR-143/145 target, and inducer of VSMC proliferation. While Jag-1/Notch signaling regulates miR-143/145 transcription independent of SRF, full acquisition of the contractile phenotype requires both SRF and miR-143/145.

There are limited data on the regulation of vascular miR such as miR-143/145, although transcriptional regulation by SRF/myocardin has been characterized in VSMC (22, 23). In certain cancers, miR-145 is transcriptionally regulated by the tumor suppressor p53 via direct binding to a putative p53 response element located in the miR-145 promoter (33). It is likely that miR-143/145 is impacted by multiple pathways in VSMC, which may provide additional control or amplification of signals leading to differentiation. Our study provides the first link between Jag-1/Notch and miR-143/145 pathways, which extends our understanding of the mechanistic signaling of Notch in VSMC (16, 34).

The miR-143/145 locus has been targeted in the mouse, and is not required for vascular development (23, 24, 35). However, altered vascular structure and morphology was reported in postnatal vessels, with pathological lesion formation (35) or abnormal response to vascular injury on the miR-143/145-null background (23). While Jag-1/Notch signaling has clear and non-redundant roles during embryonic vascular development, an additional role postnatally may be as an upstream regulator of miR-143/145 to re-establish the contractile phenotype during vascular remodeling. In this regard, it is known that Notch signaling components are induced during vascular repair (36), and can affect the outcome of pathological vascular remodeling at the level of endothelial recovery (Notch1) (37), neointimal lesion formation (Notch1) (26), and endothelial function in cardiac allografts (38). Therefore, we propose that physiological regulation of miR-143/145 by Notch signaling is relevant during vascular repair processes that require changes in the VSMC contractile phenotype.

Although both Jagged and Delta-like (Dll) families of Notch ligands activate CBF1-dependent Notch signaling in VSMC, only Jag-1/Notch signaling consistently activated the miR-143/
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145 promoter in VSMC. Future studies will determine Notch ligand and receptor specificity upstream of miR-143/145 regulation. Differences in gene regulation by the Jagged or Dll family of ligands may provide for temporally distinct responses, since endothelial cells change their ligand expression during angiogenesis (39). Based on the known link to VSMC differentiation, it seems plausible that Notch3 receptor is a likely candidate for regulating miR-143/1445 via Jag-1 signaling (15). Additionally, our present and published studies show that expression of an activated Notch1 or Notch2 receptor also recapitulates the VSMC contractile phenotype (5, 29) and therefore may also activate miR-143/145. Homotypic and heterotypic Notch signaling have been shown to be critical in defining vascular tone, maintaining vascular homeostasis, recruitment of mural cells and communication during angiogenesis (40). While Jag-1 expression in the endothelium activates Notch3 receptors in adjacent VSMC and promotes a mature contractile phenotype (15), Notch signaling between VSMC is also critical for mediating vascular remodeling after injury (41). It is likely that adjacent cell activation of VSMC Notch receptors by Jag-1 plays a pivotal role in regulating the miR-143/145 cluster and modulation of the VSMC phenotype.

In summary, we have identified the miR-143/145 cluster as a novel transcriptional target of Jag-1/Notch signaling in VSMC. Induction of these miR by Notch signaling is required for reduced proliferation and increased contractile gene expression by Jag-1/Notch signaling in VSMC. Because miR are now being considered as targets for translational opportunities for cardiovascular disease (42–44), our findings have wide application to understanding human disease.

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