Iroquois homeobox transcription factor (Irx5) promotes G1/S-phase transition in vascular smooth muscle cells by CDK2-dependent activation

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Iroquois homeobox transcription factor (Irx5) promotes G1/S-phase transition in vascular smooth muscle cells by CDK2-dependent activation. Am J Physiol Cell Physiol 311: C179–C189, 2016. First published May 11, 2016; doi:10.1152/ajpcell.00293.2015.—The Iroquois homeobox (Irx5) gene is essential in embryonic development and cardiac electrophysiology. Although recent studies have reported that IRX5 protein is involved in regulation of the cell cycle and apoptosis in prostate cancer cells, little is known about the role of IRX5 in the adult vasculature. Here we report novel observations on the role of IRX5 in adult vascular smooth muscle cells (VSMCs) during proliferation in vitro and in vivo. Comparative studies using primary human endothelial cells, VSMCs, and intact carotid arteries to determine relative expression of Irx5 in the peripheral vasculature demonstrate significantly higher expression in VSMCs. Sprague-Dawley rat carotid arteries were subjected to balloon catheterization, and the presence of IRX5 was examined by immunohistochemistry after 2 wk. Results indicate markedly elevated IRX5 signal at 14 days compared with uninjured controls. Total RNA was isolated from injured and uninjured arteries, and Irx5 expression was measured by RT-PCR. Results demonstrate a significant increase in Irx5 expression at 3–14 days postinjury compared with controls. Irx5 genetic gain- and loss-of-function studies using thymidine and 5-bromo-2’-deoxyuridine incorporation assays resulted in modulation of DNA synthesis in primary rat aortic VSMCs. Quantitative RT-PCR results revealed modulation of cyclin-dependent kinase inhibitor 1B (p27Kip1), E2F transcription factor 1 (E2f1), and proliferating cell nuclear antigen (Pcna) expression in Irx5-transduced VSMCs compared with controls. Subsequently, apoptosis was observed and confirmed by morphological observation, caspase-3 cleavage, and enzymatic activation compared with control conditions. Taken together, these results indicate that Irx5 plays an important role in VSMC G1/S-phase cell cycle checkpoint control and apoptosis.

Irx5; vascular smooth muscle cell; DNA synthesis; cell cycle; cyclin-dependent kinase 2; apoptosis

Proliferation of vascular smooth muscle cells (VSMCs) is important during vascular remodeling in atherosclerosis and restenosis (4, 36). Therapeutic strategies using various gene delivery modalities to impede cell cycle initiation and progression or promote apoptosis in VSMCs have been attempted to reduce postinjury intimal hyperplasia (10, 35).

Homeobox genes encode a family of transcription factors characterized by a 60-amino acid conserved DNA-binding motif known as the homeodomain (43). Despite their well-known effects during embryonic development, some homeobox genes are involved in pathological states such as the development of cancer (1) and atherosclerosis and restenosis after angioplasty (17, 20, 25, 26). Iroquois homeobox (Irx) genes are evolutionarily conserved from sponges to humans (32). The Iroquois gene family belongs to the TALE (3-amino-acid loop extension) superclass of atypical homeodomain-containing proteins that also contains a class-defining nine-amino acid motif termed the IRO box of unknown function (8). Six Iroquois (Irx1-6) genes in mammals and lower organisms have been shown to be involved in development (2, 19, 30, 33).

IRX5 protein has been characterized in the developing mouse embryo (6, 12, 14). In situ hybridization studies revealed the expression pattern of Irx5 in the developing heart and nervous system in mice and lower organisms (13, 14). IRX function is highly dependent on cell type and context. Studies using Irx5 null mice indicate that Irx5 is required for retinal cone bipolar cell development and formation of the cardiac ventricular repolarization gradient by direct repression of Kv4.2 K+ channel expression (11, 15). Previous clinical studies report that Irx5 expression is elevated in ventricles of patients with dilated cardiomyopathy (3). Studies in Xenopus embryos revealed that Irx5 is positively regulated by another homeodomain transcription factor, Hoxb4, that can activate the apoptotic pathway (28, 40). Transient knockout of Irx5 resulted in cell cycle arrest in the G1/M phase and subsequent apoptosis in the hyperproliferative human prostate cancer cell line LNCaP in a vitamin D3-dependent manner (31). Thus these cumulative observations imply that Irx5 might function as a cell growth regulator in adult VSMCs during proliferative vasculopathic disease progression.

Here we report that Irx5 is expressed in human and murine VSMCs and that Irx5 expression is significantly increased in response to mitogenic stimulation. The presence of IRX5 protein was elevated in VSMCs in the neointima after balloon injury in rat carotid arteries. Furthermore, enforced expression of Irx5 results in loss of G1/S-phase checkpoint control, elevation of DNA synthesis activity, and reduced cell growth rate, as well as apoptosis following S-phase arrest. Thus these results suggest that Irx5 may partially govern adult VSMC fate in the context of proliferative vascular disease.

MATERIALS AND METHODS

Rat carotid artery balloon injury. All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of the Atlanta University Center. Male Sprague-Dawley rat carotid arteries were subjected to balloon catheterization, and the presence of IRX5 was examined by immunohistochemistry after 2 wk. Results indicate markedly elevated IRX5 signal at 14 days compared with uninjured controls. Total RNA was isolated from injured and uninjured arteries, and Irx5 expression was measured by RT-PCR. Results demonstrate a significant increase in Irx5 expression at 3–14 days postinjury compared with controls. Irx5 genetic gain- and loss-of-function studies using thymidine and 5-bromo-2’-deoxyuridine incorporation assays resulted in modulation of DNA synthesis in primary rat aortic VSMCs. Quantitative RT-PCR results revealed modulation of cyclin-dependent kinase inhibitor 1B (p27Kip1), E2F transcription factor 1 (E2f1), and proliferating cell nuclear antigen (Pcna) expression in Irx5-transduced VSMCs compared with controls. Subsequently, apoptosis was observed and confirmed by morphological observation, caspase-3 cleavage, and enzymatic activation compared with control conditions. Taken together, these results indicate that Irx5 plays an important role in VSMC G1/S-phase cell cycle checkpoint control and apoptosis.

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rats (350–400 g body wt; Charles River Labs, Raleigh, NC) were anesthetized with ketamine (80 mg/kg) and xylazine (6 mg/kg) and subjected to balloon injury, as previously described (29). Briefly, an F2 Fogarty catheter was inserted into the carotid artery, inflated, and pulled back and forth six times to denude the vessel. Animals were euthanized, and thoracotomies were performed. Carotid arteries were harvested and snap-frozen or embedded in paraffin at the indicated times for total RNA isolation and immunohistochemical analysis, as previously described (29).

Tissue isolation, processing, and immunostaining. At the indicated times, rat carotid arteries were perfused with PBS for 5 min, and a 2-cm section of carotid artery distal to the aorta was excised and incubated overnight in 10% buffered formalin solution. Segments of the artery were cut into eight serial 5-μm-thick cross sections at 0.15-mm intervals, as previously described (29). Total RNA from carotid arteries was isolated, and quantitative RT-PCR was performed as described elsewhere (29). For immunohistochemical analysis, sections were rehydrated, blocked with normal serum and 0.01% Triton X-100 in PBS, and incubated with anti-IRX5 primary antibody (1:600 dilution; PAI-17056, Affinity Bioreagents, Golden, CO). Nonimmune IgG (1:600 dilution; PAI-17056, Affinity Bioreagents, Golden, CO). Nonimmune IgG (1:600 dilution) was used as a negative control. Sections were incubated with biotinylated secondary antibody and developed with diaminobenzidine (DAB Substrate Kit for Peroxidase, Vector Laboratories, Burlingame, CA) for detection. Cell nuclei were counterstained with hematoxylin, and immunohistochemical images were captured using an Olympus BX60 microscope at ×40 magnification.

Cell culture. Primary rat aortic smooth muscle cells (RASMCs) were obtained from Cell Applications (San Diego, CA). Human aortic smooth muscle cells (HASMCs) and human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Baltimore, MD). HUVECs were maintained in endothelial cell growth medium. HASMCs were maintained as previously described (29). Low-passage primary rat aortic smooth muscle cells (RASMCs) were used for this study. pAd/miR-Irx5 was used as a negative control for these studies.

Western immunoblotting. RASMCs were lysed by addition of protein extraction reagent (Pierce) with protease inhibitor cocktail (Sigma). The concentration of protein in cell lysates was determined using bicinchoninic acid (Pierce) with BSA as the standard. Protein (50 μg) was resolved on a 10% SDS-polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane, and probed with polyclonal antibodies against PCNA (1:1,000 dilution), GAPDH (1:2,000 dilution), E2F transcription factor 1 (E2F1, 1:300 dilution), and proliferating cell nuclear antigen (PCNA, 1:1,000 dilution) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Growth arrest and DNA damage-inducing protein (GADD45β and GADD45γ) antibodies were obtained from Abcam (Cambridge, MA). Protein bands were visualized with SuperSignal chemiluminescence substrate (Thermo Fisher Scientific).

Determination of DNA synthesis. [3H]Thymidine incorporation was determined as previously described with modifications (29). RASMCs were transfected with the indicated adenovirus vector [multiplicity of infection (MOI) = 200] for 8 h and replaced with growth arrest medium for 48 h. Cells were labeled with [3H]thymidine for the final 8 h of the 48-h incubation period. Total cells lysates were harvested and mixed with scintillation cocktail, and [3H]thymidine incorporation (cpm) was measured using a liquid scintillation counter. 5-Bromo-2′-deoxyuridine (BrdU) incorporation was determined according to the manufacturer’s protocols (EMD Millipore). Briefly, RASMCs were seeded at 2 × 10⁶ cells/well in a 24-well plate, incubated for 48 h, and, during the last 8 h of the incubation period, labeled with BrdU. Cells were then fixed and incubated with anti-BrdU antibody (1:100 dilution) for 1 h in multiwell plates, washed, and incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min. Colorimetric absorbance was measured in each well with a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA) at dual (450 and 540 nm) wavelengths.

Fluorescence microcytometry. For cell cycle stage analysis, RASMCs were seeded at a density of 7.5 × 10⁴ cells/well in six-well plates. After adenovirus transduction, cells were harvested at the indicated times, cells were then washed with PBS and fixed in ice-cold 70% ethanol. Fixed cells were assessed for cell cycle stage using Cell Cycle Reagent (EMD Millipore) for 30 min, and DNA content was measured using a flow cytometer. Colorimetric absorbance was measured in each well with a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA) at dual (450 and 540 nm) wavelengths.

Construction of recombinant adenovirus vectors. Replication-deficient adenovirus vectors were constructed using Gateway technology (Thermo Fisher Scientific), as previously described (29). Briefly, Ad/Irx5 was generated by using the full-length mouse Irx5 cDNA (GenBank accession no. NM_018826) from the plasmid pYX-Asc/Irx5 (Thermo Fisher Scientific). To facilitate detection of exogenous Irx5 (Thermo Fisher Scientific). To facilitate detection of exogenous Irx5 (Thermo Fisher Scientific). To facilitate detection of exogenous Irx5 (Thermo Fisher Scientific). Ad/LacZ was used as a negative control for these studies.

Caspase activity assay. Caspase-3 activity was measured using the CaspACE Assay System, Colorimetric (Promega, Madison, WI). Briefly, RASMCs were plated at 1 × 10⁴ cells per 90-mm dish. After serum deprivation, cells were treated for 6 h with Ad/LacZ, Ad/Irx5, or mock adenovirus. After removal of the virus, cells were cultured in growth medium. At the indicated times, detached and adherent cells
Table 1. Primer sequences for real-time PCR studies

| Gene       | GenBank Accession No. | Forward (5’-3’)                  | Reverse (5’-3’)                  | Product Length, bp |
|------------|-----------------------|----------------------------------|----------------------------------|-------------------|
| Human Irx5 | NM_005853.4           | ccgccgcgccttctctctctgta          | tggcgctcccttggtgctctcctcc       | 150               |
| Human β-actin | NM_001101.2         | gctgtctgtgacsaaccgct            | caaacatgtctgggtctcatctctcc       | 353               |
| Rat Irx5   | NM_001030044.1       | cgcggctgcttcaagaaga             | gacccgcctctcccgcagac            | 364               |
| Rat β-actin | NM_031144.1          | cctagggccacccgtaagaatgt         | gtcgccggccacccgcagc             | 219               |
| Rat E2f1   | XM_230765.4          | aagcgtctgacctgtggaacctg         | tagatggcccttgttgtgcttta         | 160               |
| Rat PcnA   | NM_022381.2          | caagtatactgcgggacctta           | cagtggtgtccttcggta              | 230               |
| Rat Gadd45B| NM_000100321.1       | gaggcgcccaacgtgataagtt           | gttctcggccctggtgcgc             | 226               |
| Rat Gadd45γ| NM_001076640.1       | acagtgccgccagctcctgaatgt        | gttctcccaagccgcagatgc           | 161               |
| Rat p27kip1| NM_031762.2          | gcggtgccttcaatgggtctca          | cgggcttttgggctgtcgc             | 232               |

Irx, Iroquois homeobox transcription factor; E2f1, E2F transcription factor 1; PcnA, proliferating cell nuclear antigen; Gadd, growth arrest and DNA damage; p27kip1, cyclin-dependent kinase inhibitor 1B.

were harvested. The cell lysates were centrifuged at 15,000 × g for 20 min at 4°C. The cleared supernatant was transferred to a new micro-centrifuge tube for measurement of total protein concentration and diluted to the same concentration. The assay was performed in a total volume of 100 μl in a 96-well plate. For measurement of caspase-3 activity, 20 μl of the lysate (100 μg of total protein) from each sample were used. The plate was incubated at 37°C for 4 h. Absorbance at 405 nm in the wells was measured using the spectrophotometer (SpectraMax 190, Molecular Devices). The rate of color change was normalized to the protein concentration. In some experiments the cell lysates were incubated with the caspase-3 inhibitor Z-DEVD-FMK (50 μmol/l; EMD Millipore) before assessment of caspase activity.

Statistical analysis. Data reflect independent experiments that were performed at least three times with similar results. Values are means ± SD. Data were evaluated by t-test or one-way ANOVA. When the overall F test of the ANOVA was significant, a multiple-comparison test was used. *P < 0.05; **P < 0.01.

Fig. 1. Iroquois homeobox transcription factor (Irx5) expression in peripheral vasculature and under mitogenic conditions. A: representative images of RT-PCR products from human aortic smooth muscle cells (HASMCs), human umbilical vein endothelial cells (HUVECs), rat aortic smooth muscle cells (RASMCs), and carotid artery tissue were resolved in 2% agarose gel, and expected-size bands were observed and compared with β-actin loading control [human and rat β-actin (hβ-actin and rβ-actin)]. Irx5 and rIrX5, human and rat Irx5. B: quantitative RT-PCR assessment of endogenous Irx5 expression in human and rat vascular smooth muscle cells (hVSMCs and rVSMCs), HUVECs, and carotid artery tissue. Data are expressed as fold change vs. day 0 or 0 h. *P < 0.05; **P < 0.01.
(Tukey’s) test was applied to determine the sources of differences, which were considered statistically significant when $P \leq 0.05$.

**RESULTS**

*Irx5 is expressed in cells of the peripheral vasculature under mitogenic conditions.* To determine the presence of *Irx5* mRNA in the vasculature, we used RT-PCR to examine endogenous *Irx5* expression in cultured VSMCs, endothelial cells, and adult rat artery. Results in Fig. 1A demonstrate that *Irx5* mRNA was present in cultured HASMCs, HUVECs, RASMCs, and intact rat carotid arteries. To determine the relative expression level of *Irx5* in the vasculature, we examined *Irx5* expression in cultured VSMCs, endothelial cells, and adult rat carotid artery by quantitative RT-PCR. Our results (Fig. 1B) demonstrate that *Irx5* mRNA was present in cultured human VSMCs, HUVECs, rat VSMCs, and rat carotid arteries. Moreover, the expression level of *Irx5* was significantly higher in VSMCs than vascular endothelial cells and carotid arteries. It is well known that the proliferative capacity of adult VSMCs under normal conditions is reduced in vivo; we examined the expression of *Irx5* in the context of serum deprivation or stimulation in RASMCs. Results in Fig. 1C indicate that expression of endogenous *Irx5* in rat VSMCs was significantly decreased by serum deprivation after 24 h and was maintained over a period of 3 days. Addition of serum resulted in a significant increase in *Irx5* expression (Fig. 1D) as early as 6 h after serum stimulation, which was maintained over a period of 24 h.

**IRX5 protein signal is increased in the neointima during proliferative vascular remodeling.** To examine IRX5 protein in the context of vascular injury, adult rat carotid arteries were subjected to balloon catheterization injury and examined for the presence of IRX5 protein by immunohistochemistry after 14 days. Results in Fig. 2A indicate positive staining for IRX5 in a population of VSMCs in the medial layer of uninjured arteries. However, we observed markedly elevated positive staining for IRX5 in VSMCs within the neointima, medial layer, and endothelium in injured arteries compared with uninjured control arteries. Notably, IRX5-positive staining was observed mainly within the nuclei of cells located in the neointima. However, some background staining was also observed in the cytoplasmic compartment in the medial layer of injured and uninjured arteries. No significant staining was observed in arteries stained with IgG2a isotype control anti-

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**Fig. 2.** IRX5 immunohistochemistry during proliferative vascular remodeling. **A:** representative images of IRX5 immunostaining in balloon-injured and uninjured control rat carotid arteries show increased IRX5 staining in VSMCs in the neointima 14 days postinjury. IRX5-positive staining is present in medial, neointimal, and endothelial layers in injured arteries at 14 days postinjury; some background staining is present in the cytoplasm. No significant staining was observed in IgG control vessels. IRX5 and IgG primary antibody dilution was 1:600. Cell nuclei were counterstained with hematoxylin. Magnification ×40; scale bars = 20 μm. **B:** quantitative RT-PCR assessment of *Irx5* expression in total RNA isolated from balloon-injured rat carotid arteries ($n \geq 5$) at 0–14 days postinjury. Data indicate a significant increase in *Irx5* expression as early as 3 days postinjury. *Irx5* expression was normalized to β-actin and is shown as fold change vs. uninjured control. *$P < 0.05$; **$P < 0.01$ vs. control.*
body. To examine the temporal expression pattern of Irx5 during proliferative vascular remodeling, we performed quantitative RT-PCR experiments using RNA samples extracted from balloon-injured rat carotid arteries at 3 h to 7 days postinjury. Our results demonstrate a significant increase in Irx5 expression as early as 3 days (Fig. 2B), which was maintained at 3–14 days postinjury compared with uninjured contralateral carotid arteries (control). No significant difference in Irx5 temporal expression was observed in control arteries over the 14-day period. Taken together, these results indicate that Irx5 mRNA and IRX5 protein are present in the peripheral vasculature in vitro and in vivo and are significantly increased during VSMC proliferative remodeling.

Irx5 promotes cellular DNA synthesis in VSMCs. To investigate IRX5 function in VSMCs, we generated gain-of-function (Ad/Irx5) and loss-of-function (Ad/miR-Irx5) recombinant adenovirus vectors. RASMCs were transduced (MOI = 200) with Ad/Irx5-expressing mouse IRX5 with a conditional V5 fusion epitope located at the COOH-terminal region. Results in Fig. 3A demonstrate adenovirus vector-mediated ectopic Irx5 expression after 48 h, which was partially inhibited upon cotransduction with Ad/miR-Irx5 (MOI = 100). Previous reports indicate a role for Irx5 in cell cycle regulation and apoptosis. Therefore, we investigated IRX5 function in DNA synthesis. RASMCs were transduced with Ad/Irx5, Ad/LacZ, or mock adenovirus. Cells were then serum-deprived for 48 h, with addition of [3H]thymidine during the last 8 h. Results indicate that [3H]thymidine incorporation and BrdU incorporation (Fig. 3, B and C) were significantly increased in Ad/Irx5-transduced VSMCs compared with VSMCs transduced with Ad/LacZ control and mock adenovirus.

Next, we performed transient Irx5 knockdown studies using a synthetic microRNA targeted to endogenous Irx5 mRNA (Ad/miR-Irx5) to examine the effects on DNA synthesis in RASMCs. Our results (Fig. 3D) indicate a significant reduction of Irx5 mRNA abundance in RASMCs transduced with Ad/miR-Irx5 as early as 12 h posttransduction compared with Ad/miR-Neg vector and mock control conditions. Similarly, we observed a significant reduction of BrdU incorporation (Fig. 3E) in Ad/miR-Irx5-transduced cells compared with Ad/miR-Neg- and mock-transduced cells in normal growth conditions. These data indicate a functional role of IRX5 during DNA synthesis in VSMCs during quiescence and during proliferation in vitro.

Ectopic Irx5 expression promotes S-phase entry. Our previous results indicate that ectopic expression of Irx5 results in an

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Fig. 3. IRX5 promotes DNA synthesis. A: Western immunoblot of V5 epitope-tagged IRX5. RASMC protein lysates were harvested 48 h posttransduction, and Western blot analysis was performed with anti-V5 antibody (1:5,000 dilution). Transduction with Ad/Irx5 (multiplicity of infection (MOI) = 200) alone or with Ad/miR-Irx5 (MOI = 100) results in overexpression or knockdown, respectively, of Irx5-V5 fusion protein. Anti-rat β-actin (1:5,000 dilution) was used as loading control. B and C: Ad/Irx5 expression in quiescent RASMCs results in a significant increase in DNA synthesis compared with control conditions. RASMCs were incubated in serum-deprived medium for 48 h and labeled with [3H]thymidine or 5-bromo-2′-deoxyuridine (BrdU) during the last 8 h. D and E: In RASMCs, transduction with Ad/miR-Irx5 (MOI = 200) under normal growth conditions results in a significant reduction of endogenous Irx5 expression (D) and BrdU incorporation (E). No significant differences were observed in control conditions. *P < 0.05; **P < 0.01.

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increase in DNA synthesis; therefore, we performed cell cycle analysis to determine if IRX5 function affects cell cycle stage distribution under mitogenic conditions. RASMCs were transduced (MOI = 200) with Ad/Irx5 or Ad/miR-Irx5 or the control vector Ad/LacZ or Ad/miR-Neg, respectively, for 8 h. Cells were growth-arrested for 24 h and then cultured in normal growth conditions for 48 h. Results from cell cycle analysis (Fig. 4A) indicate that cells transduced with Ad/Irx5 transitioned from the G1 to the S phase as early as 8 h. Moreover, RASMCs in the S-phase were maintained at significantly higher levels (Fig. 4B) at 8–48 h compared with mock- and Ad/LacZ-transduced cells. Transient knockdown of endogenous Irx5 resulted in a significant reduction of the S-phase population at 24–48 h compared with Ad/miR-Neg-transduced cells. Interestingly, the proportion of RASMCs in the G2/M phase was not significantly different in any of the adenovirus vector-transduced conditions compared with mock-transduced cells. Viable cell count analysis (Fig. 4C) indicates a significant reduction of Irx5-transduced cells compared with control LacZ-transduced RASMCs. Transient knockdown of endogenous Irx5 resulted in a significant reduction of cell numbers compared with control miR-Neg-transduced cells.

IRX5-induced S-phase entry is mediated by repression of P27Kip1 and CDK2 activation. The CDK2 inhibitor P27Kip1 has been well reported as a negative regulator of cell cycle progression at the G1/S-phase checkpoint (23). To examine the expression level of p27Kip1 in Irx5-transduced cells, quiescent RASMCs were transduced with Ad/Irx5 or Ad/LacZ. At 0–48 h, total RNA was harvested and p27Kip1 expression was examined by quantitative RT-PCR. Results in Fig. 5, A and B, demonstrate a significant reduction of p27Kip1 expression and P27Kip1 protein levels in Irx5-transduced cells after 12 h compared with Ad/LacZ control conditions. Furthermore, pharmacological inhibition (Fig. 5C) of CDK2 activity with purvalanol A in Ad/Irx5-transduced RASMCs resulted in a significant reduction of cells in the S phase after 48 h compared with vehicle-treated cells. Therefore, these data suggest that ectopic expression of Irx5 results in early repression of p27Kip1 and subsequent loss of G1/S-phase cell cycle checkpoint control that is dependent on CDK2 activity.

Ectopic Irx5 expression results in an increase in the S-phase mediators E2f1 and Pcn1. Previous reports have characterized E2f1 as an essential mediator of cell cycle progression in VSMCs (38, 39). Therefore, we examined the expression level of E2f1 in RASMCs transduced with mock adenovirus, Ad/

![Image](https://example.com/image1.png)

![Image](https://example.com/image2.png)

Fig. 4. IRX5 increases the population of VSMCs in the S phase under normal growth conditions. Genetic gain or loss of Irx5 modulates the percentile population of RASMCs in the S phase of the cell cycle. RASMCs were transduced with adenovirus vector (MOI = 200) and cultured under normal growth conditions. Cells were harvested, and cell cycle DNA content was analyzed for percent cell population at all stages of the cell cycle (A), percent S-phase population at 0–48 h (B), and number of viable RASMCs in each condition (C). *P < 0.05; **P < 0.01 vs. control vector.
IRX5 promotes G1/S-phase transition in VSMCs. Based on the observation that IRX5-transduced RASMCs resulted in an aberrant entry into the S phase, we examined if ectopic expression of IRX5 also induces an apoptotic phenotype. To determine if cells were initiating apoptosis, we transduced quiescent RASMCs with Ad/Irx5 or Ad/LacZ and incubated them in normal growth medium. Morphological changes characteristic of cells undergoing apoptosis were observed, with cell rounding, shrinkage, cleavage, and condensation of chromatin by Hoechst 33342 staining after 48 h (Fig. 7A). Induction of apoptosis by Irx5 was also evidenced by cleavage of procaspase-3, as observed by the appearance of the large (17/19-kDa) fragment of activated caspase-3 (Fig. 7B). To further evaluate apoptosis, we examined caspase-3 activity in RASMCs transduced with Ad/Irx5, Ad/LacZ, or mock adenovirus. Our results demonstrate that caspase-3 activity was significantly increased in a dose- and time-dependent manner (Fig. 7C) and that the caspase-3 inhibitor Z-DEVD-FMK (40 μmol/l) significantly inhibited IRX5-mediated caspase-3 activity. Moreover, RASMCs transduced with Irx5 resulted in a significant increase in expression and protein levels of the DNA damage checkpoint control genes Gadd45β and Gadd45γ compared with control conditions (Fig. 8). Taken together, these results indicate a novel function of IRX5 to induce cell cycle control at the G1/S-phase entry transition, which is demonstrated by a reduction of p27kip1 expression and CDK2 activation. Furthermore, we observed an IRX5-mediated increase in DNA synthesis and S-phase entry, with subsequent apoptosis, as indicated by elevated expression of the Gadd45β and Gadd45γ DNA damage repair genes.

Irx5, or Ad/LacZ and incubated in serum-deprived medium for 48 h. Results demonstrate a significant increase in E2f1 expression and E2F1 protein levels (Fig. 6, A and B) in cells transduced with Ad/Irx5 at 24 h posttransduction. Similarly, we observed a significant increase in Pcna1 expression and PCNA protein (Fig. 6, A and C), a known gene target of E2f1, compared with Ad/LacZ and mock control conditions.

IRX5 induces an apoptotic phenotype in VSMCs. Based on the observation that Irx5-transduced RASMCs resulted in an aberrant entry into the S phase, we examined if ectopic expression of Irx5 also induces an apoptotic phenotype. To determine if cells were initiating apoptosis, we transduced quiescent RASMCs with Ad/Irx5 or Ad/LacZ and incubated them in normal growth medium. Morphological changes characteristic of cells undergoing apoptosis were observed, with cell rounding, shrinkage, cleavage, and condensation of chromatin by Hoechst 33342 staining after 48 h (Fig. 7A). Induction of apoptosis by Irx5 was also evidenced by cleavage of procaspase-3, as observed by the appearance of the large (17/19-kDa) fragment of activated caspase-3 (Fig. 7B). To further evaluate apoptosis, we examined caspase-3 activity in RASMCs transduced with Ad/Irx5, Ad/LacZ, or mock adenovirus. Our results demonstrate that caspase-3 activity was significantly increased in a dose- and time-dependent manner (Fig. 7C) and that the caspase-3 inhibitor Z-DEVD-FMK (40 μmol/l) significantly inhibited IRX5-mediated caspase-3 activity. Moreover, RASMCs transduced with Irx5 resulted in a significant increase in expression and protein levels of the DNA damage checkpoint control genes Gadd45β and Gadd45γ compared with control conditions (Fig. 8). Taken together, these results indicate a novel function of IRX5 to induce cell cycle control at the G1/S-phase entry transition, which is demonstrated by a reduction of p27kip1 expression and CDK2 activation. Furthermore, we observed an IRX5-mediated increase in DNA synthesis and S-phase entry, with subsequent apoptosis, as indicated by elevated expression of the Gadd45β and Gadd45γ DNA damage repair genes.
DISCUSSION

In recent years, molecular linkages between cell death, cell survival, and the cell cycle have become an object of intense research (23). The phosphoinositide 3-kinase/Akt pathway, cyclins, CDKs, CDK inhibitors, p53, retinoblastoma protein/E2F pathway, Myc family, and Bcl-2 family were reported to have partial cross-utilization in opposing cell physiological processes such as the cell cycle, apoptosis, and cell survival/proliferation. Here we report the novel function of Irx5 to promote the G1/S-phase transition of the cell cycle, suppress proliferation, and, ultimately, induce apoptosis in adult VSMCs.

IRX5 has been previously reported to function in the embryonic development of different tissues and organs. Recent reports describe Irx5 expression in the adult heart and functions to establish the cardiac ventricular repolarization gradient (9, 15, 34). We report, for the first time, Irx5 expression in the mammalian vasculature in vitro and in vivo. Previous reports have characterized the homeodomain protein Gax as an antiproliferative mediator in VSMCs (44). In contrast, our data demonstrate that endogenous expression of Irx5 is responsive to serum treatment, suggesting that Irx5 may function as a proproliferative mediator. It will be interesting to examine in detail the function of IRX5 during proliferative vascular remodeling in vivo.

During our Irx5 gain-of-function experiments, we observed a loss of G1/S-phase checkpoint control and aberrant induction of S-phase entry followed by apoptotic cell death in VSMCs. A recent study that characterizes transient depletion of Irx5 in LNCaP cells reports blockade of the G2/M phase of the cell cycle followed by apoptosis (31). We also observed apoptosis in our experiments; however, we did not observe a significant increase in the population of cells in the G2/M phase. We also observed a significant reduction of the population of VSMCs in the S phase after transient knockdown of Irx5 using a synthetic microRNA. In contrast, we did not observe a significant decrease in p21 or p53 expression in our Irx5 gain-of-function studies. This may be due to differences in the growth characteristics of the cells utilized and/or the presence of cell-specific transcription cofactor expression in LNCaP epithelial cells.
compared with VSMCs. Indeed, Irx5 null mice are viable, although they demonstrate a number of phenotypic differences compared with their wild-type counterparts.

Enforced expression of Irx5 resulted in VSMC detachment and nuclear fragmentation, which, in combination with other characteristic markers, serve to distinguish apoptosis from cell senescence or inflammatory-necrotic modes of death (20, 37). To further substantiate the induction of apoptosis by Irx5, we examined the levels of cleaved caspase-3 and caspase-3 activity after transduction with Irx5. We found that overexpression of Irx5 in VSMCs resulted in elevated levels of both cleaved caspase-3 formation and activity in a dose-dependent manner. Thus, Irx5 appears to activate downstream effectors of apoptotic death. Of note, activation of caspases and cell death became apparent only after 36 h, which indicates that Irx5 expression, entrance of the arrested VSMCs into the G1/S phase, and transition to the S phase are required before the cells can engage in the apoptosis signaling cascade. It is unclear if IRX5 directly or indirectly regulates expression of proapoptotic mediators and should be rigorously investigated.

Previous studies indicate that the vitamin D receptor can function as a cofactor in Irx5-mediated transcription regulation (31). Walker et al. (41) demonstrated that heterodimerization of Irx4 with the vitamin D receptor is essential for spatial repression of the slow myosin heavy chain 3 (slow MyHC3) gene in the ventricle of the adult heart. Costantini et al. (15) reported that the cardiac corepressor mBOP was required for Irx5-mediated repression of Kv4.2 K+ channel gene (Kcnq2) expression in ventricular cardiac myocytes. The presence of the cofactor mBOP in the endo- vs. epicardial layer of the heart is essential in the establishment of a proper epicardial K+ gradient function under normal conditions. Therefore, it is likely that other cell-specific cofactors may determine whether IRX5 functions to transactivate, or repress, target gene expression in vitro and in vivo. Furthermore, Wang et al. (42) reported that the hetero- and homodimerization of IRX proteins also function to modulate gene expression in vitro. These reports suggest that transcription cofactor(s) complexes and other histone modifier proteins such as histone deacetylases, may be required for Irx5-mediated induction of S-phase entry in VSMCs. However, cell-specific cofactors that may associate with IRX5 during VSMC proliferation remain to be determined.

Previous reports suggest that IRX proteins act mainly as transcriptional repressors that recognize and bind a unique palindromic motif (15, 18, 22). Thus, the signaling mechanism of IRX5 effects may rely on other downstream target genes. Our results demonstrate that Irx5-induced S-phase entry was associated with a significant reduction of p27kip1 expression, which has been well reported to promote cell death through loss of G1/S-phase cell cycle checkpoint control (27, 38). Our experiments demonstrate that the CDK2 inhibitor purvalanol A blocked IRX5-induced S-phase entry, suggesting a possible IRX5 functional dependence on CDK2 activity through direct or indirect repression of p27kip1. Similarly, dysregulation of E2f1 expression results in S-phase arrest and subsequent apoptosis mediated by p53 in many cell types, including VSMCs (7). Our additional results demonstrate that IRX5-induced S-phase entry and apoptosis were associated with upregulation of E2f1 during mitogenic stimulation in VSMCs.

In the context of DNA damage checkpoint control, Xenopus Iroquois (XIRO) homeoproteins were reported to coordinate the cell cycle by regulating Gadd45γ expression in Xenopus (16). In a recent study using gene expression profiling, Rosati et al. (34) reported that increased levels of Irx5 are associated with deregulation of the cell cycle and apoptosis mediators GADD45β, BRCA1, and Cdkn2a in the hearts of miRNA1-2 null mice, suggesting that regulation of these molecules was mediated by elevated Irx5 expression. Similarly, we observed an Irx5-mediated induction of Gadd45β and Gadd45γ expression in aortic VSMCs. This observation indicates that the S-phase entry and apoptosis induced by Irx5 in VSMCs are associated with induction of DNA damage. Whether Irx5 can modulate expression of these genes directly, or indirectly, is unknown.

In the present study we demonstrate that Irx5 is expressed in VSMCs. Forced expression of Irx5 in adult VSMCs resulted in a loss of G1/S-phase checkpoint control, with subsequent apoptosis, in conjunction with modulated expression of cell cycle and DNA damage mediators. Given the critical role of

Fig. 8. Effect of IRX5 on expression of DNA damage checkpoint control genes. Gadd45β and Gadd45γ mRNA (A and C) and protein (B) were dramatically increased in Irx5-transduced cells. Total RNA and protein were isolated from Ad/Irx5, Ad/LacZ- (MOI = 200), or mock-transduced RASMCs under normal growth conditions at 0–48 h, and quantitative RT-PCR and Western immunoblotting were performed. Irx5 relative expression levels were normalized to β-actin. Data are expressed as fold change vs. 0 h. *P < 0.05; **P < 0.01 vs. control vector.
VSMC growth and migration in proliferative vascular diseases, IRX5 may be an important target in the development of future therapeutic strategies to prevent postinjury vascular stenosis and other VSMC proliferative remodeling complications.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
D.L., V.P., and L.M.A. performed the experiments; D.L., V.P., and L.M.A. analyzed the data; D.L., M.B., and L.M.A. interpreted the results of the experiments; D.L. and L.M.A. prepared the figures; D.L., M.B., and L.M.A. drafted the manuscript; D.L., M.B., and L.M.A. edited and revised the manuscript; D.L., V.P., M.B., and L.M.A. approved the final version of the manuscript; M.B. and L.M.A. developed the concept and designed the research.

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