Hypoxia-induced miR-1260b regulates vascular smooth muscle cell proliferation by targeting GDF11

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**Materials and Methods**

**Cell culture**

Human primary pulmonary artery smooth muscle cells (PASMCs) were purchased from Lonza (CC-2581) and cultured as previously described (11). For hypoxia, PASMCs in Sm-Gm2 medium (Lonza) were grown in a sealed modular incubator chamber (Billups-rothenberg Inc.) for 24 h at 37°C after flushing with a mixture of 5% CO₂, 1% O₂ and 94% N₂ for 4 min. For GDF11 stimulation, 10 ng of recombinant GDF11 purchased from R&D Systems was used.

**Quantitative reverse transcriptase-PCR (qRT-PCR)**

Real-time PCR was performed for quantitative analysis of the change in levels of mRNA or miRNA expression. Levels of mRNAs were normalized to 18S rRNA. The primers employed in this study were as follows: 18S rRNA, 5’-GTAACCGTGGACCCATT-3’ and 5’-CCATCCAATCGGTAGTCG-3’; GDF11, 5’-ACCACCGAGACCGCTTATTAG-3’ and 5’-GGGTACAGGCCGTAGTGTTA-3’; CBX6, 5’-GAACACCCGGTATAGGGAAGA-3’ and 5’-GGGTACAGGCCGTAGTGTTA-3’; CHTF8, 5’-ATGGAGCTACAGGGAGGAGT-3’ and 5’-CTGATCCCAGGAGGTTGTT-3’; DGCR2, 5’-CTGAGATGTGTTGCTGCTG-3’ and 5’-CAGCAGTGACAGGGAGGAGT-3’; MAFG, 5’-CGACCGTGGACTTTTGTATGTTGAGA-3’; NFIC, 5’-ACGTGACATACGACCTGAAC-3’ and 5’-GGGTACAGGCCGTAGTGTTA-3’; POLR2F, 5’-CGACCGTGGACTTTTGTATGTTGAGA-3’ and 5’-GCTCGCTCTCGTACTTGCTT-3’; TMEM104, 5’-CTGAGATGTGTTGCTGCTG-3’ and 5’-AGTAGACGCCACCCCAAAC-3’; and UBE2H, 5’-CTGATCCCAGGAGGTTGTT-3’ and 5’-CCACCCAATAAGGAAAC-3’ and 5’-TTCTGCTTCCAGCTCCTCCTC-3’; NFIC, 5’-ACGTGACATACGACCTGAAC-3’ and 5’-GGGTACAGGCCGTAGTGTTA-3’; POLR2F, 5’-CGACCGTGGACTTTTGTATGTTGAGA-3’ and 5’-GCTCGCTCTCGTACTTGCTT-3’; TMEM104, 5’-CTGAGATGTGTTGCTGCTG-3’ and 5’-AGTAGACGCCACCCCAAAC-3’; and UBE2H, 5’-TGAAGCGAGTTGAAAGAAG-3’ and 5’-CCACCCAATAAGGAAAC-3’ and 5’-TTCTGCTTCCAGCTCCTCCTC-3’; NFIC, 5’-ACGTGACATACGACCTGAAC-3’ and 5’-GGGTACAGGCCGTAGTGTTA-3’; POLR2F, 5’-CGACCGTGGACTTTTGTATGTTGAGA-3’ and 5’-GCTCGCTCTCGTACTTGCTT-3’; TMEM104, 5’-CTGAGATGTGTTGCTGCTG-3’ and 5’-AGTAGACGCCACCCCAAAC-3’; and UBE2H, 5’-TGAAGCGAGTTGAAAGAAG-3’ and 5’-CCACCCAATAAGGAAAC-3’. We used miScript SYBR Green PCR kit and miScript primer assays from Qiagen to measure the levels of mature miR-1260b expression. The levels of U6 small nuclear RNA as the control were measured to calculate the relative expression level of miR-1260b.
Data was analyzed using a comparative C_T method in Bio-Rad software. All procedures were carried out at least three times and the average results with standard errors are presented.

Transfection of miRNAs

The miR-1260b mimic and negative control miRNA were purchased from Genolution Pharmaceuticals. PASMCs were transfected with 5 nM miRNA mimics using RNAi Max (Invitrogen) according to the manufacturer's protocol. The hsa-miR-1260b inhibitor (anti-miR-1260b) was purchased from Ambion and was transfected at 50 nM using G-fectin (Genolution Pharmaceuticals) according to the manufacturer's instructions.

RNA interference

For knockdown of GDF11, small interfering RNAs (siGDF11) synthesized by Genolution Pharmaceuticals were used. The target sequence of siGDF11 was 5’-CAAUGACAAGCAGCAGAUUAU-3’. As a control, negative control siRNA from Genolution Pharmaceuticals was used.

Luciferase reporter constructs

A part of the 3'UTR sequence of GDF11 and a GDF11 3'UTR sequence containing mutations within the predicted MRE1 were cloned into the pIS0 vector (Addgene) that included the luciferase gene. To amplify the 3'UTR sequence of GDF11, RT-PCR was performed using mRNA isolated from PASMCs. The primers used were 5’-TTAGAGCTCCGTGTGCAATACAACAGAGG-3’ and 5’-ATTGGCCGGGCCTCACATCTCTTTACACT-3’. For the 3'UTR mutant construct
including mutated MRE sequences, an upstream region that contained a mutated sequence in MRE1 and a downstream region that contained a mutated sequence in MRE3 were amplified separately using primers containing the \textit{XhoI} restriction site. Two PCR products were cleaved by \textit{XhoI} restriction enzyme and ligated. The ligated DNA fragment was subsequently cloned into the pIS0 vector. To amplify the upstream region, 5'-ATGGAGCTCGGGAGGAGCAGGACCTATTGAGGG-3' and 5'-TTCCTCGAG CTTGCTCTCTACCTGCTTGCTC-3' were used. To amplify the downstream region, 5'-ATGCTCGAGAGGATTCTGGAAGGGGGACA-3' and 5'-ATTGGCCGCACTGCATCTCCTTCACT-3' were used. Each predicted MRE sequence was cloned into the pIS0 vector. The primers were as follows: MRE1, 5'-CAACAGAGGGAGCAGCTGGGAAACGG-3' and 5'-TTCCCACC TGCCTCCCTCTGTTGAGCT-3'; MRE2, 5'-AGCTCCCATGCGGGGGTGGGAG CCGG-3' and 5'-CTCCCACCCTGGGAGCTAGCT-3'; and MRE3, 5'-GAGCCACAGACCAAGGTGGGAACCGG-3' and 5'-TTCCCACC TTGGTCTGCTGGCTCAGCT-3'.

\textbf{Luciferase assay}

Cotransfection of control or miR-1260b mimics and luciferase reporter constructs into Cos7 cells was performed using Lipofectamine 2000 (Life technologies). As an internal transfection control, a $\beta$-galactosidase expression plasmid was used. After 24 h, the luciferase activities were measured and shown after normalization to the $\beta$-galactosidase activity.

\textbf{Western blot}
Total cell lysates were prepared in TNE buffer (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, and 0.1 mM EDTA), and proteins were separated in SDS-PAGE and transferred onto PVDF membranes. Transferred membranes were probed for GDF11 (Abcam, ab71347), β-actin (Santa Cruz, sc47778), phospho-Smad 3 (Cell Signaling) and Smad 3 (Cell Signaling) antibodies, followed by anti-rabbit secondary antibodies from Santa Cruz Biotechnology, Inc.

**Immunofluorescence staining**

Equal amounts of PASMCs placed in chamber well slides were transfected with miR-1260b, control mimic or siGDF11. The cells were fixed using 2% paraformaldehyde in PBS and incubated with 3% BSA in PBS to block unspecific binding of the antibodies. After permeabilization in 0.1% Triton X-100 in PBS, the cells were probed for Ki-67 antibody (Abcam, #ab16667), followed by goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, #A-11008). Hoechst 33342 (Thermo Fisher Scientific, #62249) was used to stain nuclei. Images were obtained using a Zeiss Axio Imager Z1 microscope. We counted at least 2000 cells per condition and show the percentages of Ki-67 positive cells. The graphs showed the average ± S.E. of triplicate experiments.

**Trichloroacetic acid (TCA)-induced protein precipitation**

PASMCs were transfected with control or miR-1260b for 48 h. Media was collected and mixed with one volume of 20% TCA. After precipitation, the pellet was washed with cold acetone and dissolved in 1X SDS sample buffer.
Statistical Analysis

All experimental procedures were carried out in triplicate and analyzed with unpaired Student’s $t$-test. Data are presented as the average with standard errors. Statistical significance is defined as $p < 0.05$. 
