




miR-128-3p Is a Novel Regulator of Vascular Smooth Muscle Cell Phenotypic Switch and Vascular Diseases

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RATIONALE: MicroRNAs (miRNAs, miRs) are small noncoding RNAs that modulate gene expression by negatively regulating translation of target genes. Although the role of several miRNAs in vascular smooth muscle cells (VSMCs) has been extensively characterized, the function of miRNA-128-3p (miR-128) is still unknown.

OBJECTIVE: To determine if miR-128 modulates VSMC phenotype and to define the underlying mechanisms.

METHODS AND RESULTS: We screened for miRNAs whose expression is modulated by an altered DNA methylation status in VSMCs, and among the hits, we selected miR-128. We found that miR-128 was expressed in various tissues, primary murine cells, and pathological murine and human vascular specimens. Through gain- and loss-of-function approaches, we determined that miR-128 affects VSMC proliferation, migration, differentiation, and contractility. The alterations of those properties were dependent upon epigenetic regulation of key VSMC differentiation genes; notably, Kruppel-like factor 4 was found to be a direct target of miR-128 and able to modulate the methylation status of the pivotal VSMC gene myosin heavy chain 11 (*Myh11*). Finally, in vivo lentiviral delivery of miR-128 prevented intimal hyperplasia in a mouse model of carotid restenosis without modifying vital cardiovascular parameters.

CONCLUSION: miR-128 is a critical modulator of VSMCs and is regulated by epigenetic modifications upon stress. Its modulation in the context of disease could be exploited for therapeutic purposes.

VISUAL OVERVIEW: An online [visual overview](#) is available for this article.

Key Words: atherosclerosis ■ carotid artery injuries ■ microRNA ■ vascular diseases ■ vascular remodeling

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Vascular smooth muscle cells (VSMCs) are key cellular components of arteries, playing a fundamental role in vessel homeostasis by maintaining vascular wall tone and integrity.^{1,2} VSMCs exhibit phenotypic plasticity: they can switch from a contractile/nonproliferating phenotype to a migratory/proliferating one in response to extracellular stimuli or environmental cues.³ VSMC identity is defined by the expression of specific markers, such as smooth-muscle α actin (*Acta2*), smooth muscle myosin heavy chain (*Myh11*), transgelin (*Sm22*), and

calponin (*Cnn1*). In cardiovascular diseases, the differentiation status of VSMCs is altered,^{4,5} and the expression of these specific VSMC biomarkers is modulated.⁶

MicroRNAs (miRNAs, miRs) are small noncoding RNAs regulating gene expression mainly by decreasing mRNA translation. Their key role in modulating VSMC phenotypic switch in vitro and in vivo has been proven.^{7,8} For instance, the miR-143/miR-145 cluster is highly enriched in differentiated VSMCs,^{9–11} and the expression of these 2 miRNAs is reduced in pathological vascular

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Novelty and Significance

What Is Known?

- Vascular smooth muscle cell (VSMC) physiology and homeostasis are regulated by different epigenetic mechanisms.
- MicroRNAs (miRNA) are known to regulate vascular disease development.
- Modulation of microRNA-128-3p (miR-128) is involved in cardiomyocyte proliferation.

What New Information Does This Article Contribute?

- miR-128 expression is associated with hypomethylation of the myosin heavy chain 11 (*Myh11*) promoter.
- miR-128 modulates VSMC phenotypic switching by directly controlling KLF4 (Kruppel-like factor 4) activity and, hence, expression of *Myh11*.
- Ectopic expression of miR-128 is beneficial in a mouse carotid artery stenosis model.

In this study, we identified several miRNAs modulated by DNA methylation in VSMCs. Among those, we selected one—namely miR-128—that is conserved at the level of the precursor sequence also in humans, identifying it as a putative new regulator of VSMC physiology. Indeed, miR-128 controls VSMC migration, proliferation, and contractility, and its expression is reduced in diseased arteries in mice and humans. Mechanistically, miR-128 directly controls KLF4, which in turn positively modulates methylation of the promoter of the key VSMC gene *Myh11*, thereby controlling VSMC differentiation and phenotypic switching. Therapeutic targeting of miR-128 in a mouse model of vascular stenosis improved disease outcome. These findings provide insights into the role of miR-128 in the regulation of vascular pathophysiology.

Nonstandard Abbreviations and Acronyms

3' UTR	3' untranslated region
ACTA2	smooth-muscle actin
AGO2	argonaute 2
Ang	angiotensin II
CTR	control cells
GO	Gene Ontology
miR, miRNA	microRNA
PDGF-BB	platelet-derived growth factor BB
PRKD1	protein kinase D1
UHRF1	ubiquitin-like containing PHD and RING finger domains 1
VSMC	vascular smooth muscle cell

specimens.^{12,13} However, their modulation is able to hinder pathological stimuli, such as atherosclerosis,¹⁴ and the consequences of vascular stenosis.^{12,13} Other non-VSMC-restricted miRNAs could also play a crucial role in VSMC homeostasis.^{15–17}

On this last point, miRNA-128-3p (miR-128) is one of the most abundant and enriched miRNAs in the adult brain,^{18–20} and its role in muscular cells, including cardiomyocytes²¹ and myoblasts,²² has been demonstrated. For instance, Huang et al. showed that miR-128 modulates cardiomyocyte proliferation and function by targeting the chromatin modifier SUZ12 (polycomb repressive complex 2 subunit).²¹ However, the role of this miRNA in VSMC biology remains unexplored.

Here, we demonstrate that miR-128 plays an essential role in the regulation of VSMC differentiation. Gain- and

loss-of-function in vitro and in vivo experiments revealed that this miRNA directly modulates VSMC function by targeting KLF4 (Kruppel-like factor 4) and then regulating the DNA methylation level of the key VSMC differentiation marker myosin heavy chain 11 (*Myh11*).

METHODS

Detailed experimental procedures, mouse models of vascular dysfunction, and cell culture are described in the Supplemental Materials in the [Data Supplement](#). Please see the Major Resources Table in the [Data Supplement](#). Primers for real-time quantitative polymerase chain reaction (RT-qPCR) assay are presented in Table III in the [Data Supplement](#).

Data Availability

All other data are included within the article or Supplementary Information or available from the authors on request.

RESULTS

miR-128-3p Expression Is Modulated in Differentiated VSMCs

To explore miRNAs potentially modulated by the VSMC DNA methylation status, phylogenetically conserved and expressed in muscular tissues, we performed a screening approach based on 3 sequential steps: (1) first, we used RT-qPCR to profile the expression of murine miRNAs, comparing cells transduced with 2 different short hairpin RNAs (shRNAs) targeting the epigenetic master regulator UHRF1 (ubiquitin-like containing PHD and RING finger domains 1)²³ with cells treated with a

scrambled sequence (shSCR). Among the statistically modulated miRNAs, we selected only those upregulated at least 1.5× by both shUHRF1 (short harpin RNA vs UHRF1) sequences. (2) Then, using the miRNAviewer tool (<http://people.csail.mit.edu/akiezun/microRNA-viewer/index.html>), we selected only the miRNAs with a conservation rate—calculated on the precursor sequence of 50 different species—>97 %. (3) Finally, the data was integrated with the expression levels of the miRNAs selected in step 2 in human tissues (<https://ccb-web.cs.uni-saarland.de/tissueatlas/>). This process identified 7 candidates enriched in muscle (variance stabilizing normalization expression >2; Figure 1A, Figure 1A and Supplemental file I in the [Data Supplement](#)). The top hit of this list was miR-29b-3p, the role of which has already been extensively studied in VSMC biology,^{24–26} and, similarly, data are already available for miR-26a-5p,²⁷ miR-142a-3p,²⁸ and let-7g-5p.²⁹ We, therefore, decided to investigate the role of another highly expressed miRNA, miR-128, although, in the future, it would be interesting also to study miR-381-3p in the same cellular context.

Analysis of different tissues confirmed that miR-128 was expressed in muscular tissues, including mouse aorta (Figure 1B and Figure 1B in the [Data Supplement](#)) and human popliteal artery (Figure 1C and Figure 1C in the [Data Supplement](#)). Measurement of miR-128 in different primary cells demonstrated its expression in all contractile cell types, including explanted mouse aortic VSMCs (Figure 1D). As miR-128 expression is directly linked to cell differentiation,^{18,21,22} we tested whether a similar correlation was present also in VSMCs. Indeed, when a phenotypic switch toward a contractile phenotype was induced by maintaining cells in a quiescent condition (with 0.1 % FBS; Figure 1IA and 1IB in the [Data Supplement](#)), expression of miR-128 was increased compared with proliferative, undifferentiated cells (kept in 10 % FBS; Figure 1E). In addition, by measuring primary miR-128 (pri-miR-128) expression in the quiescent condition, we confirmed that miR-128 is transcriptionally modulated in VSMCs (Figure 1IC in the [Data Supplement](#)).

PDGF-BB (platelet-derived growth factor BB) is a well-known modulator of VSMC phenotypic plasticity and a potent atherogenic stimulus that triggers VSMC dedifferentiation, proliferation, and migration.³⁰ Therefore, to assess if miR-128 is modulated during VSMC phenotypic switching, cells were exposed to PDGF-BB (Figure 1IB and 1ID in the [Data Supplement](#)): as expected, expression of miR-128 was reduced in PDGF-BB-treated cells compared with controls (Figure 1F). miR-128 expression was also determined in VSMCs exposed to hypoxia.³¹ Interestingly, also this stimulus reduced miR-128 expression (Figure 1G) and modulated VSMC differentiation (Figure 1IE in the [Data Supplement](#)), but it did not affect VSMC proliferation (Figure 1IF in the [Data Supplement](#)), probably due to some potential compensatory mechanism that could merit a further investigation.

Next, the expression of miR-128 was determined in pathological murine and human specimens. A strong reduction of miR-128 expression was observed in the aortas of ApoE^{−/−} mice fed a cholesterol-rich diet (Western diet) as compared to aortas of mice on a normal diet (Chow diet; Figure 1H). A similar finding was obtained in human popliteal arteries with evidence of aneurysm (Figure 1I and Table I in the [Data Supplement](#)), a condition in which VSMC phenotypic switching has been already demonstrated.³²

Altogether, these findings indicated that the expression of miR-128 is altered during VSMC phenotypic transition in vitro and in vivo and were suggestive of a role for miR-128 in vascular diseases.

miR-128 Overexpression Significantly Decreases VSMC Proliferation, Migration, Electrophysiological Properties, and Contractility

miR-128 has been shown to act as an oncosuppressive miRNA that impairs cell proliferation in different primary tumors.³³ Thus, we decided to investigate whether miR-128 could exert a similar effect in VSMCs. To this end, primary VSMCs were transduced with a lentiviral vector to overexpress miR-128 (Figure 1IIA in the [Data Supplement](#)), and proliferation and migration then evaluated. Growth curve (Figure 2A) and bromodeoxyuridine incorporation assays (Figure 2B) demonstrated that miR-128 negatively modulates cell proliferation. In line with this observation, miR-128-overexpressing VSMCs had a reduced S phase and a significant increase in the G0/G1 phase (Figure 2C), indicating decreased cell growth. In addition, a wound-healing assay revealed reduced migratory capacity in miR-128-overexpressing VSMCs compared with control cells (CTR; Figure 2D). These results were then compared with those obtained with 2 benchmark VSMC miRNAs: miR-143 and miR-145. Remarkably, the modulation of VSMC properties induced by miR-128 was greater than that evoked by miR-143 or miR-145 (Figure 1IIB through 1IIF in the [Data Supplement](#)). When overexpressed, miR-128 promoted VSMC cytoskeletal rearrangement, as demonstrated by the increase in the number of cells displaying highly organized smooth-muscle actin (ACTA2) stress fibers (Figure 2E and 2F). This structural change was associated with an increased cell size, assessed by calculating cell area (Figure 2G) and perimeter (Figure 1IIIG in the [Data Supplement](#)). The effects of miR-128 on cellular morphology strongly suggest that this miRNA regulates the differentiation status of VSMCs. Analysis of VSMC differentiation markers, such as Cnn1, Sm22, Myh11, and Acta2, by RT-qPCR (Figure 2H) and Western blotting (Figure 2I) supported this notion. Other genes critical for VSMC differentiation, including serum transcription

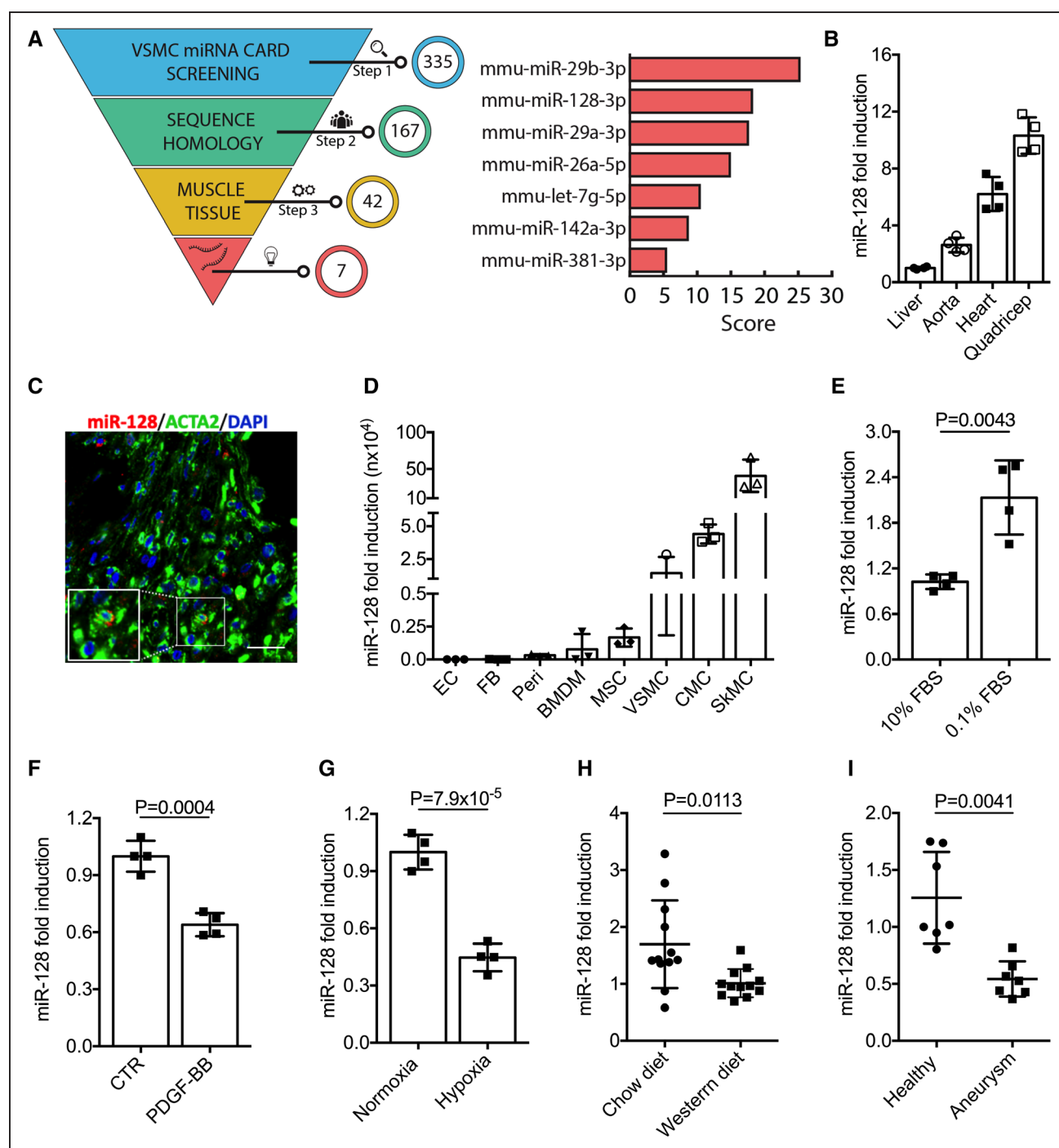


Figure 1. MicroRNA (miRNA)-128-3p (miR-128) expression is modulated in vascular smooth muscle cell (VSMC) differentiation.

A, Schematic representation of the procedure for the identification of putative miRNAs modulated by alteration of VSMC methylation status. Detailed information is provided in the Methods section. **B**, Real-time quantitative polymerase chain reaction (RT-qPCR) evaluation of miR-128 expression in contractile female mouse tissues, compared with liver (noncontractile one; $n=4$); no statistical comparison has been performed. **C**, In situ hybridization for miR-128 in human aneurytic vessels ($n=4$). Scale bar: 100 μ m. **D**, RT-qPCR evaluation of miR-128 expression in cells obtained from murine tissue ($n=3$; endothelial cells [EC], fibroblasts [FB], pericytes [Peri], macrophages [BMDM], mesenchymal stem cells [MSC], vascular smooth muscle cells [VSMC], cardiomyocytes [CMC], and skeletal muscle cells [SkMC]); no statistical analysis has been performed. **E–G**, RT-qPCR evaluation of miR-128 expression in VSMCs treated for 24 h with serum deprivation (0.1% FBS; **E**, $n=4$), PDGF-BB (platelet-derived growth factor; **F**, $n=4$), and hypoxia (**G**, $n=3$; 1% O_2). **H–I**, RT-qPCR evaluation of miR-128 expression in aortic samples of ApoE^{−/−} mice fed a Chow or Western diet (**H**, $n=11$); and aneurytic human popliteal arteries and relative controls (**I**, $n=7$). Data represent the mean \pm SD. For **H** and **I**, data normality was calculated with Kolmogorov-Smirnov test. To compare means, unpaired 2-tailed Student *t* test was used in **E**, **F**, **G**, **H** while paired two-tailed Student *t* test in **I**. ACTA2 indicates smooth muscle α actin; and DAPI, 4',6-diamidino-2-phenylindole.

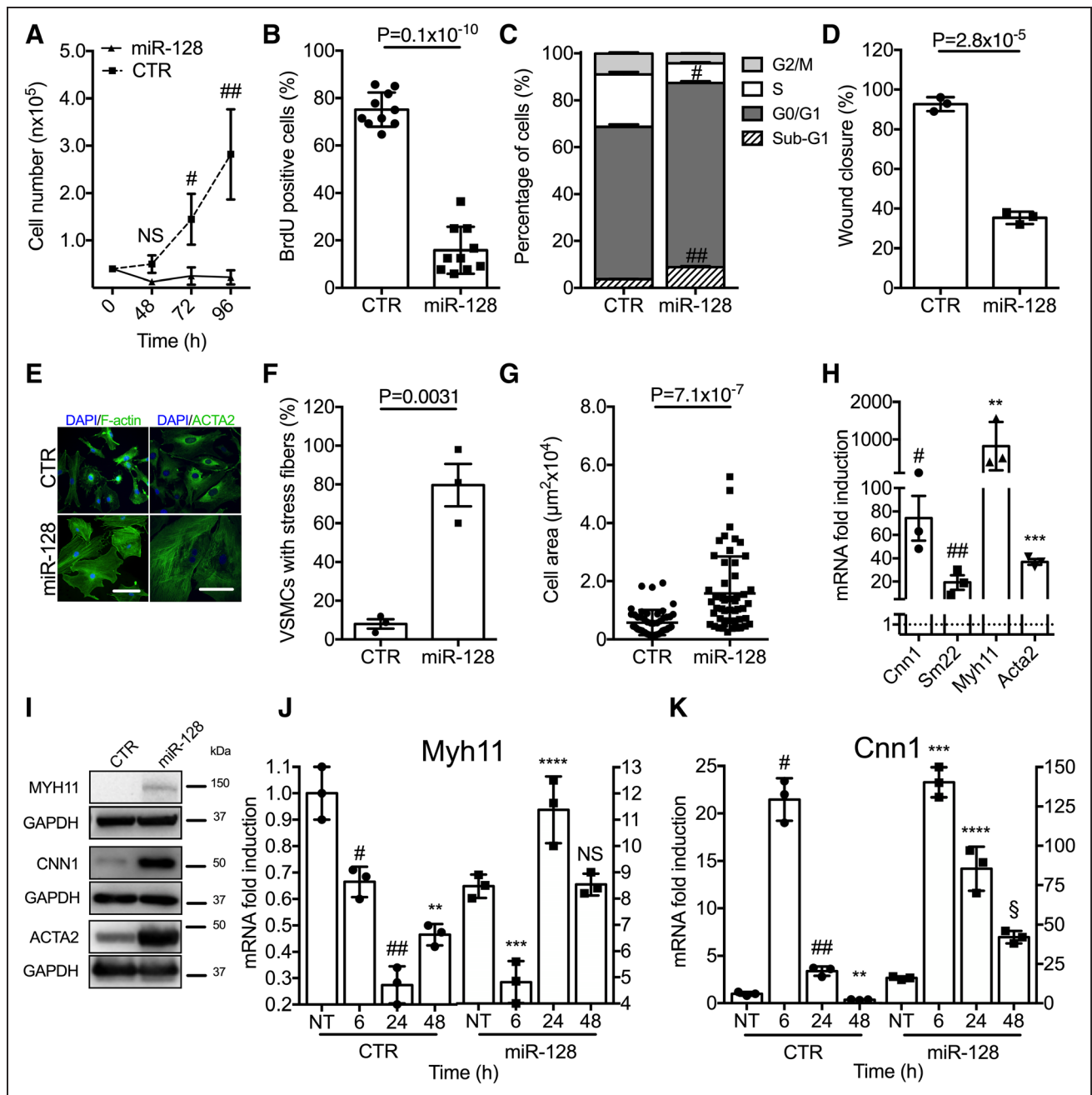


Figure 2. MicroRNA-128-3p (miR-128) regulates vascular smooth muscle cell (VSMC) plasticity in vitro.

A, Growth curve of VSMCs stably expressing miR-128 compared with control cells (CTR; n=3). **B**, Proliferation rate measured by bromodeoxyuridine (BrdU) incorporation, in CTR and miR-128-overexpressing VSMCs (n=10). **C**, Cell cycle analysis of miR-128-overexpressing cells vs CTR cells (n=3). **D**, Wound-healing assay on miR-128-overexpressing VSMCs (n=3). **E**, Staining of primary murine VSMCs overexpressing miR-128 vs CTR cells for either F-actin (left) or ACTA2 (smooth muscle α actin; right; Representative images chosen for similarity to the global quantification; Scale bar: 50 μ m) with relative quantification of stress fibers (**F**, n=3); and cell area (**G**, n=5). **H**, Expression of VSMC differentiation markers in miR-128-overexpressing VSMCs vs CTR (dotted line), measured by real-time quantitative polymerase chain reaction (RT-qPCR; n=3). **I**, Representative Western blots showing differentiation markers (Myh11 [myosin heavy chain 11], Cnn1 [calponin] and ACTA2) expression in miR-128-overexpressing VSMCs vs CTR. **J** and **K**, RT-qPCR of VSMC differentiation markers Myh11 (**J**) and Cnn1 (**K**) in CTR and miR-128-overexpressing (miR-128) VSMCs exposed to 10% FBS after 24 h of serum deprivation (FBS 0.1%, NT; n=3). Data represent the mean \pm SD. To compare means, 2-way ANOVA with Sidak multiple comparisons test was used in **A**; 1-way ANOVA with Holm-Sidak multiple comparisons test was used in **J** and **K**; 2-tailed Student *t* test was used in **B–H**. For **A**, #Adjusted *P* (Adj *P*)=0.0084, ##Adj *P*=2.5 \times 10⁻⁶; for **C**, #*P*=0.0038, ##*P*=0.004; for **H**, data were compared with CTR (represented by the dotted line, no bar present): #*P*=0.018, ##*P*=0.004, ****P*=0.047, *****P*=0.0001; for **J** and **K** data were compared with the relative NT cells (CTR or miR-128), **J**, #Adj *P*=0.0011, ##Adj *P*=3.6 \times 10⁻⁵, ***Adj *P*=7.9 \times 10⁻⁵, ****Adj *P*=0.0017, ****Adj *P*=0.0046; **K**, #Adj *P*=0.4 \times 10⁻¹¹, ##Adj *P*=3.7 \times 10⁻⁵, *Adj *P*=0.042, ***Adj *P*=3.4 \times 10⁻⁷, ****Adj *P*=1.9 \times 10⁻⁶, §Adj *P*=0.0064. DAPI indicates 4',6-diamidino-2-phenylindole; and N.S., not statistically significant.

factor (Srf)³⁴ and the cofactor myocardin (Myocd)³⁵ were also significantly increased (Figure IIIH in the [Data Supplement](#)).

We then evaluated whether miR-128 might prevent VSMC dedifferentiation when contractile cells (kept in 0.1 % FBS) are cultured again in a proliferative medium (10 % FBS). Of note, miR-128–overexpressing VSMCs maintained a contractile phenotype, as measured by RT-qPCR, regardless of the medium (Figure 2J and 2K).

Finally, we tested whether miR-128 was able to alter the electrophysiological and contractile properties of VSMCs. Patch-clamp experiments showed that miR-128–overexpressing VSMCs had a significantly more hyperpolarized membrane potential compared with CTR (Figure 3A and Figure IV in the [Data Supplement](#)). When VSMCs were exposed to a contractile stimulus, such as Angiotensin II, membrane potentials resulted to be more depolarized in comparison to cells not exposed to Angiotensin II, and the depolarization was much higher in miR-128–overexpressing cells (30.49% versus 20.75%; Figure 3A and B). A contractile assay with a collagen plug demonstrated the contractile/differentiated phenotype of miR-128–transduced VSMCs both at basal level and after Angiotensin II treatment (Figure 3C) with the latter showing a greater contractile force compared with CTR Angiotensin II cells (20.75% versus 7.74%). Furthermore, patch-clamp data confirmed also that the size of miR-128–overexpressing VSMCs was indeed greater than CTR, as indicated by membrane capacity measurements (Figure 3D).

These findings confirmed a role for miR-128 in regulating VSMC phenotypic switch.

miR-128 Overexpression Modulates the Phenotype of Other Mesodermal Cells Similarly to VSMCs

To investigate whether miR-128 modified gene expression in other cell types similar to that observed in VSMCs, we overexpressed it in fibroblast (3T3), cardiomyocyte (HL1), and endothelial (H5V) murine cell lines. Surprisingly, all the tested miR-128–transduced cell types acquired a VSMC-like morphology (Figure 3E and Figure V in the [Data Supplement](#)). This result was corroborated by RT-qPCR analysis of specific VSMC differentiation genes, which revealed their increase in all transduced cells tested (Figure 3F through 3H). This finding strongly suggests that miR-128 may act as a master switch of the gene program independently of cell context.

Moreover, electrophysiological measurements confirmed that, with the exception of endothelial cells, miR-128–overexpressing cells had a hyperpolarized membrane potential (Figure 3I) and increased membrane capacity (Figure 3J), just like VSMCs overexpressing miR-128.

Altogether, these findings strongly suggest a general effect of miR-128 in inducing a VSMC-like phenotype in cells of mesodermal origin.

Normalization of miR-128 Levels Rescues VSMC Phenotype

To strengthen the evidence on miR-128 being able to regulate VSMC phenotype, we performed rescue experiments on miR-128–overexpressing VSMCs in which the level of the miRNA was efficiently knocked-down with a miR-128 inhibitor oligonucleotide (i128; Figure VIA in the [Data Supplement](#)). Exposure to i128 was indeed able to normalize VSMC proliferation (Figure 4A and Figure VIB in the [Data Supplement](#)), migration (Figure 4B), differentiation (Figure 4C), stress fiber formation (Figure 4D), and size (Figure 4E and Figure VIC in the [Data Supplement](#)).

These gain-of-function and rescue experiments clearly demonstrate the importance of miR-128 in modulating VSMC phenotype.

Identification of KLF4 as a Target of miR-128 in VSMCs

Having determined the importance of miR-128 in regulating proliferation and differentiation of VSMCs, we searched for critical direct targets that could explain miR-128's biological effects. First, in silico analysis was employed to compare the results of the target-identification algorithms of 2 miRNA target-prediction bioinformatics programs (PITA [https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html] and Targetscan [http://www.targetscan.org/vert_72/]) with results from AGO2 (argonaute 2) RNA immune-precipitation (CLIP) performed on C2C12 cells,³⁶ a cell line in which miR-128 expression is strongly induced during differentiation (Figure VIIA and Supplemental File II in the [Data Supplement](#)). Among the resulting targets, KLF4 caught our attention (Figure 5A and 5B) because its key role in VSMC differentiation is well known³⁷ and because its level is reported to be regulated by miR-128, albeit in the cancer setting.³⁸

Because analysis of the *Klf4* 3' untranslated region (3' UTR) sequence suggested compatibility with miR-128 binding (Figure VIIB in the [Data Supplement](#)), we decided to validate this hypothesis through 3' UTR luciferase assays. To this end, luciferase constructs were generated with the 3' UTR of either *Klf4* or another putative target, *Prkd1* (protein kinase D1; Figure VIIC in the [Data Supplement](#)). We found that miR-128 bound the seed sequence of the *Klf4* 3' UTR, but not that of *Prkd1*, a finding strongly suggestive of KLF4 being a direct target of miR-128 (Figure 5C). Direct interaction between the two RNAs was also confirmed by AGO2

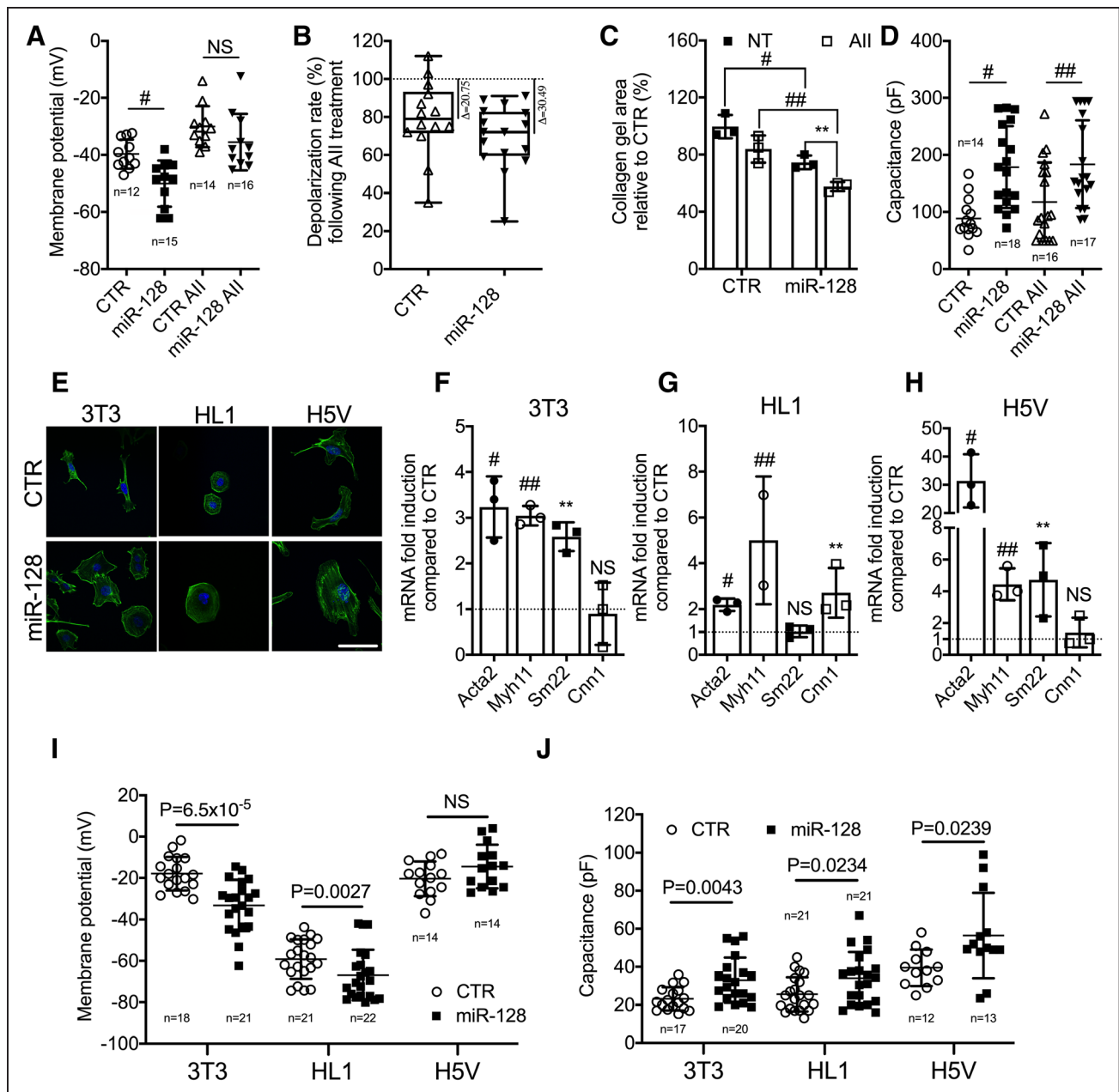


Figure 3. Molecular and functional effects of microRNA-128-3p (miR-128) upregulation on mesodermal cells.

A, Resting membrane potential (RMP) data recorded from control cells (CTR) and miR-128-overexpressing vascular smooth muscle cells (VSMCs) and CTR and miR-128-overexpressing VSMCs exposed to Ang (angiotensin II). **B**, RMP changes obtained from CTR and miR-128-overexpressing VSMCs exposed to Ang. Values are normalized to corresponding NT averages. To note a 30.49% depolarization of miR-128-overexpressing VSMCs compared with 20.75% of CTR. **C**, Quantification of collagen contraction after 24 h in Ang-treated cells, compared with NT (n=3). **D**, Membrane capacitance (Cm) data recorded from CTR and miR-128-overexpressing VSMCs and CTR and miR-128-overexpressing VSMCs exposed to Ang. **E**, Phalloidin staining of 3T3, HL1, and H5V miR-128-overexpressing cells vs CTR (representative images chosen for similarity to the global quantification; n=3). Scale bar: 50 μ m. **F–H**, Expression of VSMC differentiation markers in miR-128-overexpressing 3T3 (**F**), HL1 (**G**), and H5V (**H**) vs CTR (dotted line), measured by real-time quantitative polymerase chain reaction (n=4). **I–J**, RMP (**I**) and Cm (**J**) data recorded from 3T3, HL1, and H5V miR-128-overexpressing cells vs CTR. Data represent the mean \pm SD. Number of independent experiments is listed in the panels. To compare means, 1-way ANOVA with Tukey multiple comparisons test was used in **A**, **C**, and **D**; no test was run for **B**; 2-tailed Student *t* test was used in **F–J**. For **A**, #Adj *P*=0.016; for **C**, #Adj *P*=0.0096, ##Adj *P*=0.0054, **Adj *P*=0.048; for **D**, #Adj *P*=0.0018, ##Adj *P*=0.021; for **F**, #*P*=0.0044, ##*P*=4.5 \times 10⁻⁵, ***P*=0.0009; for **G**, #*P*=0.0015, ##*P*=0.037, ***P*=0.030; for **H**, #*P*=0.005, ##*P*=0.004, ***P*=0.048. NS indicates not statistically significant.

immunoprecipitation: indeed, we observed an enrichment of the *Klf4* 3' UTR in the pulled-down RNA from miR-128-overexpressing VSMCs compared with CTR

(Figure 5D). Coherently, overexpression of miR-128 in VSMCs induced downregulation of *Klf4* RNA and protein (Figure 5E and 5F).

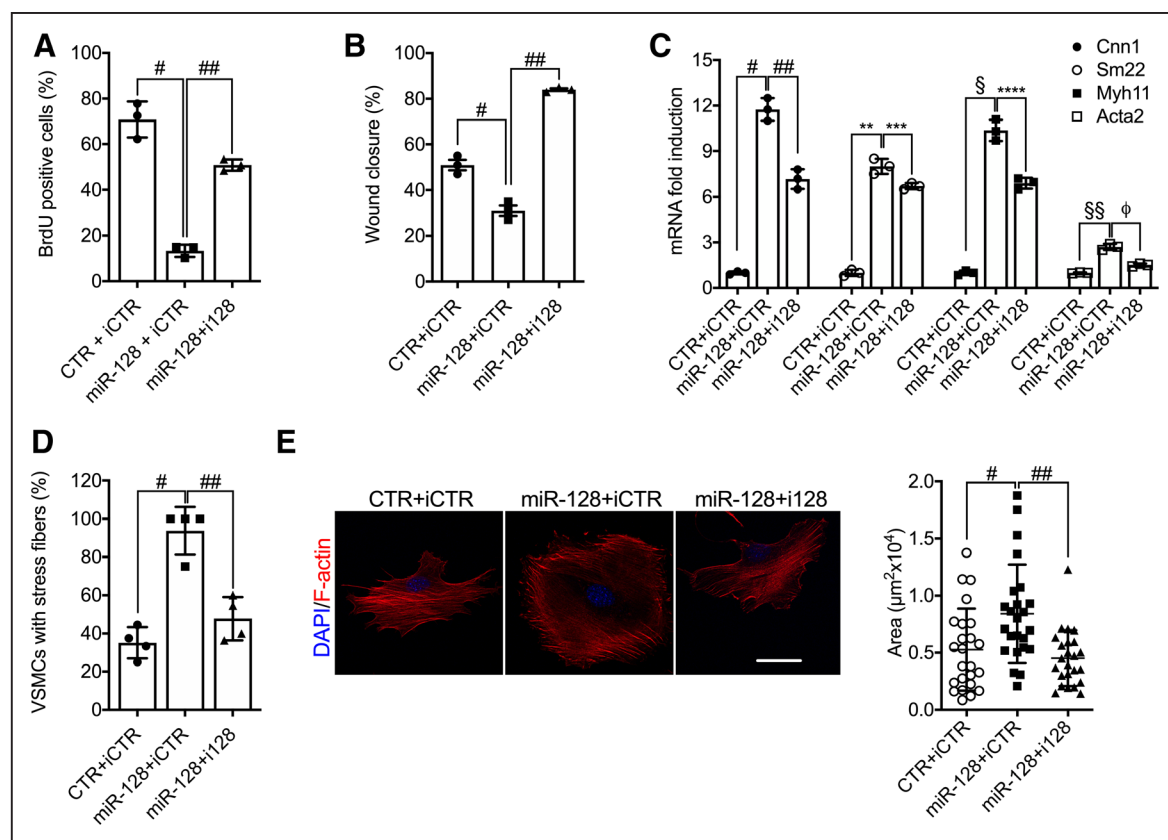


Figure 4. Inhibition of microRNA-128-3p (miR-128) restores vascular smooth muscle cell (VSMC) phenotypic features.

A, Proliferation assay measured by bromodeoxyuridine (BrdU) incorporation ($n=3$), and **(B)** Residual wound area 24 h postscratch ($n=3$) in miR-128-overexpressing VSMCs transfected with miR-128 inhibitor (i128) or control cells (CTR) inhibitor (iCTR) compared with CTR cells transfected with the iCTR. **C**, Expression of VSMC differentiation markers measured by real-time quantitative polymerase chain reaction ($n=3$). **D**, Stress fiber quantification in treated VSMCs ($n=4$). **E**, Phalloidin staining of miR-128-overexpressing VSMCs transfected with the oligonucleotides i128 or iCTR vs CTR cells (**left**), and relative quantification of stress fibers cellular area (representative images chosen for similarity to the global quantification; μm^2 ; $n=4$). Scale bar: 10 μm . Data represent the mean \pm SD. To compare means, 1-way ANOVA with Tukey multiple comparisons test was used in **A–E**; for **A**, #Adj $P=2 \times 10^{-5}$, ##Adj $P=0.0002$; for **B**, #Adj $P=0.0008$, #Adj $P=2.6 \times 10^{-6}$; for **C**, #Adj $P=1.2 \times 10^{-6}$, ##Adj $P=0.0002$, **Adj $P=7.4 \times 10^{-7}$, ***Adj $P=0.0071$, ****Adj $P=8.8 \times 10^{-7}$, §Adj $P=0.0002$, §§Adj $P=7.8 \times 10^{-5}$, ¶Adj $P=6.6 \times 10^{-5}$; for **D**, #Adj $P=8 \times 10^{-5}$, ##Adj $P=0.0005$; for **E**, #Adj $P=0.0085$, ##Adj $P=0.0009$.

These findings clearly show that miR-128 directly regulates KLF4 expression.

miR-128 Overexpression Modulates the VSMC Gene Expression Profile

To shed light on the specific molecular mechanisms in which miR-128 is involved, we performed gene expression analysis on miR-128-overexpressing VSMCs. The Illumina murine array for total RNA revealed 1284 perturbed genes, of which 701 were downregulated and 583 upregulated (using an adjusted $P < 0.01$ as the threshold for significance; Figure 6A and 6B). Hierarchical cluster analysis of the perturbed gene sets identified different patterns of Gene Ontology (GO) terms (ie, biological processes and molecular functions): response to growth factor (GO:0070848), blood vessel development (GO:0001568), and actin filament-based process (GO:0030029) were significantly enriched (Figure 6C,

top), whereas DNA replication (GO:0006260), cell division (GO:0051301), and DNA conformation change (GO:0071103) were downregulated (Figure 6C, bottom). The biological relationship among the identified GO terms was further evaluated with Metascape, in which each term is represented by a circular node with a size proportional to the number of input genes falling into that term, and a color that represents its cluster identity (GO term; Figure 6C, top and bottom, respectively). All the above-mentioned biological processes turned out to be closely interrelated, largely reflecting the change in proliferation and cellular plasticity of miR-128-overexpressing VSMCs. Cell cycle regulation, one of the most-recurrent pathways highlighted by this analysis, was validated by RT-qPCR (Figure VIIIA through VIIIC in the [Data Supplement](#)).

Taken together, these findings confirm the role of miR-128 in the regulation of VSMC differentiation status.

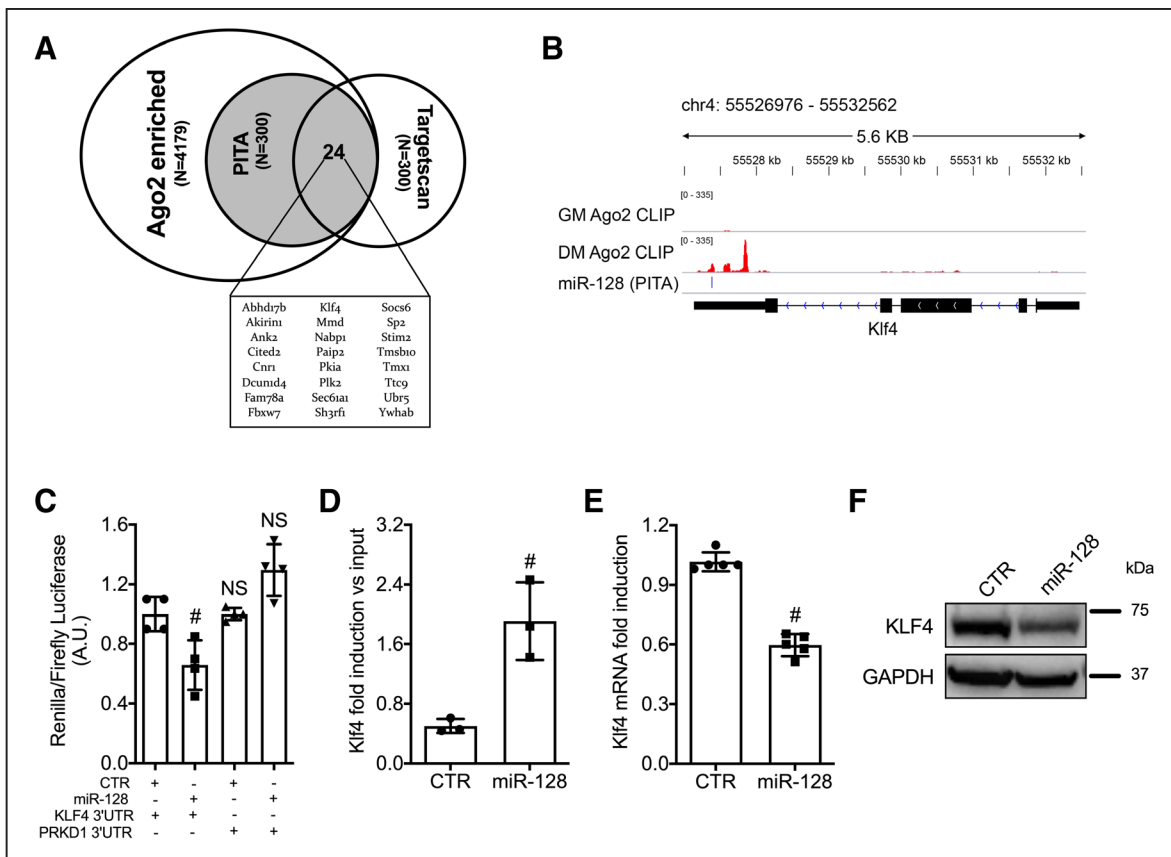


Figure 5. KLF4 (Kruppel-like factor 4) is a novel direct target of microRNA-128-3p (miR-128).

A, Schematic representation of the procedure for the identification of putative miR-128 target genes. As described in the Methods section, the analysis derives from an integration of data from a published Ago2 (argonaute 2) CLIP dataset of C2C12 cells and the miR-128 targets predicted by PITA and Targetscan softwares. **B**, Ago2-enriched reads for *Klf4* in C2C12 cells. **C**, Luciferase reporter assays using vectors encoding putative target sites in the 3' untranslated region (3'-UTR; *Klf4*, *Prkd1*). Vascular smooth muscle cell (VSMC) cells were transiently cotransfected both with miR-128 or control cells (CTR) and wild-type 3'-UTR reporter plasmids of *Klf4* or *Prkd1*. Renilla luciferase activity was measured 24 h after transfection. The results are normalized to firefly luciferase values ($n=4$). **D**, Ago2-immunoprecipitation performed on miR-128-overexpressing VSMCs vs CTR show an enrichment in *Klf4* ($n=3$). **E**, Real-time quantitative polymerase chain reaction measurement of *Klf4* expression in miR-128-overexpressing VSMCs vs CTR ($n=5$). **F**, Western blot showing the reduction of KLF4 in miR-128-overexpressing cells. Data represent the mean \pm SD. To compare means, 1-way ANOVA with Holm-Sidak multiple comparisons test was used in **C** (data compared with CTR+KLF4 3'UTR) and 2-tailed Student *t* test in **D** and **E**; for **C**, #Adj $P=0.011$; for **D**, # $P=0.01$; for **E**, # $P=1 \times 10^{-6}$. AU indicates arbitrary units; CLIP, RNA immune-precipitation; DM, differentiation medium; GM, growth medium; and NS, not statistically significant.

miR-128 Modulates VSMC Methylation Status

Having demonstrated that miR-128 modulation evokes a massive effect on differentiation in VSMCs and induces the development of a VSMC-like phenotype in other cell types, we sought to determine whether miR-128 might affect the VSMC epigenome, the assembly of chemical changes to the DNA and histone proteins in the cell. To this end, we integrated data derived from the above-mentioned gene expression profile with data from a public epigenetics database (<http://epifactors.autosome.ru>), searching for all epigenetic modulators significantly regulated in miR-128-overexpressing cells. Among the various epigenetic mechanisms, GO analysis revealed a significant downregulation of processes such as nucleosome assembly (GO:0006334), cellular response to DNA damage stimulus (GO:0006974), DNA repair (GO:0006281), histone methylation (GO:0016571), and,

in particular, regulation of methylation-dependent chromatin silencing (GO:0090308; Figure 6D and Supplemental file III in the [Data Supplement](#)).

This last finding led us to test whether miR-128 was indeed able to influence the methylation status of VSMCs. To this end, we first validated array results by measuring the expression of the DNA methyltransferase enzymes Dnmt1, Dnmt3a, Dnmt3b, and Uhrf1 in miR-128-overexpressing VSMCs, finding a significant reduction of their RNA levels compared with CTR cells (Figure 7A). In contrast, the expression of the Ten-eleven translocation family genes (Tet1, 2, and 3)—responsible for 5-hydroxymethylation of cytosine during an intermediated step in cytosine demethylation⁴⁰—was increased in the transduced cells (Figure 7B). When total 5-methylcytosine (5-mC) content was assessed, a significant reduction of the total DNA methylation status

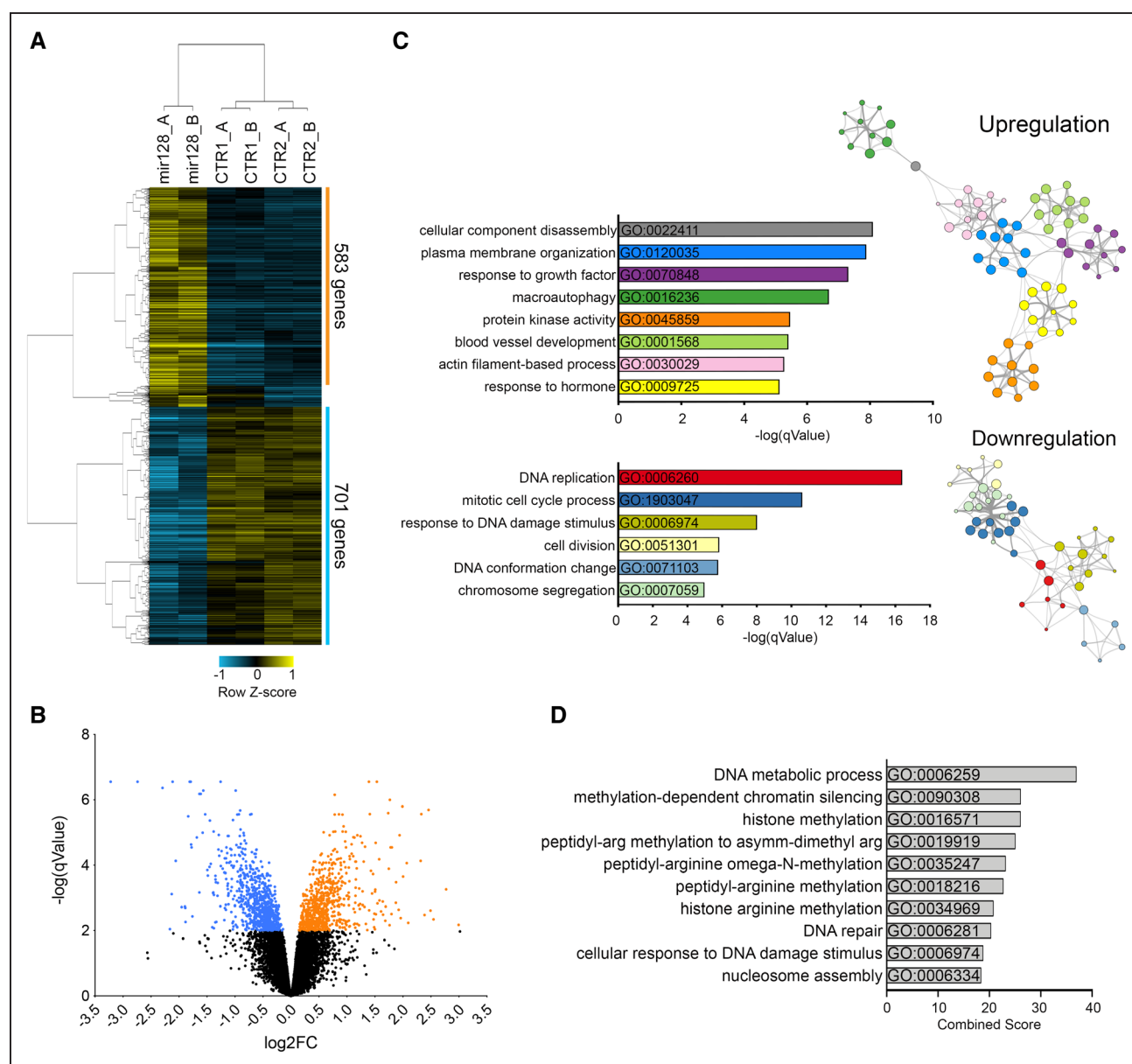


Figure 6. Differential profiling in microRNA-128-3p (miR-128)-overexpressing vascular smooth muscle cell (VSMCs) vs control cells (CTR).

A, Hierarchical clustering heat map of 1284 probes differentially expressed between miR-128 and CTR cells (Adj $P \leq 0.01$). **B**, Volcano plot, with light blue dots representing significantly downregulated protein-coding genes ($n=583$) and orange dots representing significantly upregulated protein-coding genes ($n=701$) in miR-128-overexpressing and CTR VSMCs ($q\text{ Value} = \text{adj } P\text{ value}$). **C**, Enriched gene ontology (GO) list of terms and network analysis with graphical representation relative to the upregulated (upper/left) and downregulated (lower/right) protein-coding genes. Terms with a similarity score >0.3 are linked by an edge (the thickness of the edge represents the similarity score). The network was visualized with Cytoscape (v3.1.2) in a force-directed layout and with edge bundled for clarity ($Q\text{ value} = \text{adj } P\text{ value}$). **D**, Epigenetic modulators significantly regulated in miR-128-overexpressing cells. The resulting GO terms were obtained using the enrichR combined score.³⁹

in miR-128-overexpressing cells was observed (Figure 7C), indicating that the net effect of the gene expression modifications induced by miR-128 overexpression on DNA methylation status is a decrease in overall 5-mC.

Because miR-128 overexpression modified the level of mRNAs critical for VSMC differentiation, as well as affected the global 5-mC level, we tested whether the methylation status of the promoter of their genes was affected by miR-128 expression. First, an in silico

analysis aimed at identifying the presence of CpG islands on the promoters of *Myh11*, *Acta2*, and *Sm22* (Figure VIIID in the Data Supplement) was performed. Of those, only *Myh11*—a gene whose expression was massively upregulated in miR-128-overexpressing VSMCs (Figure 3D)—was characterized by the presence of extensive CpG sequences. 5-mC chromatin immune-precipitation (Methyl-ChIP) experiments revealed a reduction of 5-mC *P* on the *Myh11* promoter in

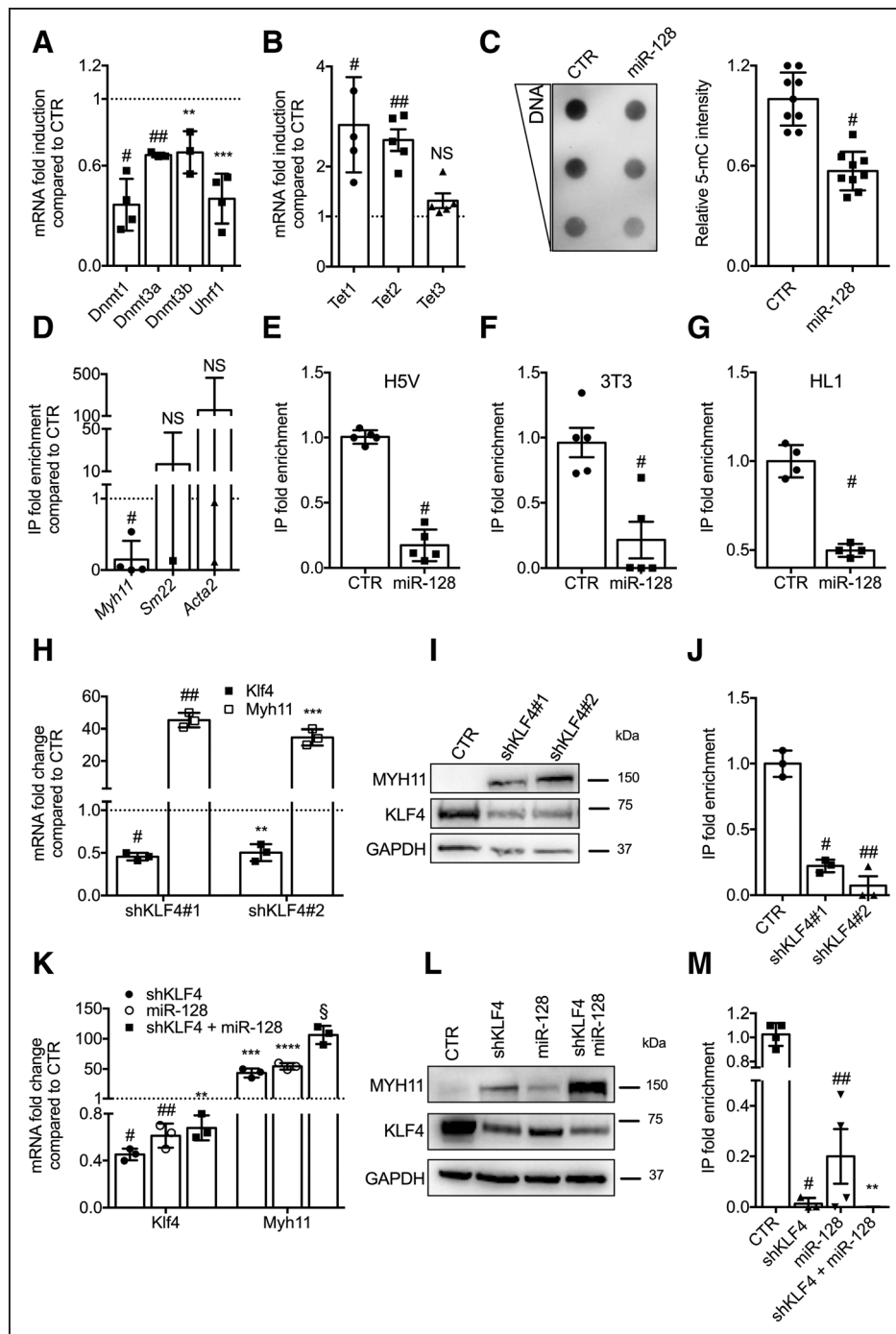


Figure 7. MicroRNA-128-3p (miR-128) affects the global methylation status of vascular smooth muscle cells (VSMCs).

A and **B**, Real-time quantitative polymerase chain reaction (RT-qPCR) showing decreased expression of *Dnmt* genes and *Uhrf1* (**A**, $n=5$) and increased expression of *Tet* genes (**B**, $n=5$) in miR-128-overexpressing VSMCs vs control cells (CTR; dotted line). **C**, Dot blot of 5-methylcytosine (5-mC) in gDNA extracted from primary mouse VSMCs overexpressing miR-128 vs CTR, and relative quantification ($n=3$). **D**, Methyl Collector assay showing reduced methylation at the myosin heavy chain 11 (*Myh11*) promoter in miR-128-overexpressing cells vs CTR ($n=4$). **E–G**, Methyl Collector assay showing reduced methylation at the *Myh11* promoter in 3T3 ($n=5$), HL1 ($n=4$), and H5V ($n=5$) in miR-128-overexpressing cells vs CTR. **H–I**, RT-qPCR (**H**) and Western Blot (**I**) in primary shKLF4-transduced VSMCs (#1, #2) compared to CTR showed the correlation with Klf4 loss and Myh11 overexpression ($n=3$). **J**, Methyl Collector assay showing the reduced methylation at the *Myh11* promoter in Klf4-silenced VSMCs vs CTR. **K–M**, Myh11 regulation when both miR-128 and Klf4 are modulated. Myh11 expression level was measured by RT-qPCR (**K**, $n=3$), Western blotting (**L**) and evaluated its promoter regulation by methyl collector assay (**M**, $n=4$). Data represent the mean \pm SD. To compare means, 2-tailed Student *t* test in **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, and **K**, while 1-way ANOVA with Holm-Sidak multicomparisons test was used in **J** and **M**; for **A**, # $P=0.003$, ## $P=0.018$, *** $P=0.009$, **** $P=0.0004$; for **B**, # $P=0.0025$, ## $P=0.0001$; for **C**, $P=6 \times 10^{-6}$; for **D**, # $P=0.0006$; for **E**, # $P=6 \times 10^{-7}$; for **F**, # $P=0.0031$; for **G**, # $P=5 \times 10^{-5}$; for **H**, # $P=0.001$, ## $P=7 \times 10^{-5}$, *** $P=0.0036$, **** $P=0.0003$; for **J**, #Adj $P=0.0069$, ##Adj $P=0.018$; for **K**, # $P=0.001$, ## $P=0.0094$, ** $P=0.018$, *** $P=0.0006$, **** $P=7 \times 10^{-5}$, \$ $P=0.0002$; for **M**, #Adj $P=3.6 \times 10^{-6}$, ##Adj $P=4.3 \times 10^{-6}$, *Adj $P=3.6 \times 10^{-6}$. IP indicates immunoprecipitation; NS, not statistically significant; Shklf, short hairpin RNA vs Klf4; and ShRNA, short hairpin RNA.

miR-128–overexpressing VSMCs (Figure 7D), a finding confirmed in the other tested mesodermic cell lines in which miR-128 triggered a VSMC-like phenotype (Figure 7E through 7G).

Because in some biological contexts KLF4 acts as a modulator of DNA methylation,^{41,42} we next determined whether this was the case also in VSMCs. We first measured the expression of *Myh11* in *Klf4*-silenced VSMCs, observing increased levels of its RNA and protein (Figure 7H and 7I). As expected, methylation of the *Myh11* promoter was reduced (Figure 7J), similarly to what we observed in miR-128–overexpressing VSMCs (Figure 7D). To further corroborate these findings, we performed experiments in which *Klf4* was silenced in miR-128–overexpressing VSMCs. Concomitant modulation of miR-128 and *Klf4* had a cumulative effect on *Myh11* RNA and protein levels (Figure 7K and 7L), as well as on the methylation level of the *Myh11* promoter (Figure 7M).

These findings strongly support the notion that the effect of miR-128 on VSMC differentiation is due, at least in part—as in the case of *Myh11*—to the regulation of specific gene promoters through direct regulation of *Klf4*.

miR-128 Overexpression Reduces Stenosis Development in Mouse Carotids

Excessive proliferation and migration of VSMCs into the intimal layer is a hallmark of post-angioplasty stenosis and of degenerative diseases of the arterial wall.⁴³ Because we found that miR-128 strongly modulates VSMC proliferation, migration, and differentiation in vitro, we wondered whether its forced expression might alter vascular physiology in vivo. We thus first evaluated the tissue distribution of systemically delivered miR-128–expressing viral vectors, observing a strong accumulation in carotid artery but not in other tissues (Figure IXA in the [Data Supplement](#)). In contrast with previous reports,^{44,45} this vascular accumulation of miR-128 did not induce senescence or calcification (Figure IXB and IXC in the [Data Supplement](#)). In addition, the assessment of cardiovascular function in miR-128–infused mice did not reveal any alteration in cardiac parameters, such as fractional shortening and ejection fraction (Figure IXD in the [Data Supplement](#)) or arterial blood pressure (Figure IXE in the [Data Supplement](#)), indicating that miR-128 delivery at baseline does not influence cardiovascular physiology.

Finally, we wondered whether miR-128 delivery to mouse artery could prevent the development of VSMC proliferation and the consequential stenosis. To test this hypothesis, we used a model of stenosis induced by the placement of a perivascular carotid collar in ApoE^{−/−} mice.²³ Four weeks after the procedure, mice were euthanized, and the stented carotids histologically analyzed. As expected, vessels treated with the control vector

showed strong intimal hyperplasia. However, delivery of the miR-128 viral vector markedly decreased neointimal area (Figure 8A and Figure XA through XF in the [Data Supplement](#)), a result indicative of reduced VSMC proliferation in vivo. Coherently, Ki-67 staining revealing that miR-128–overexpressing vessels had a reduced number of proliferating cells (Figure 8B). By contrast, there was no significant change in the number of apoptotic cells in constricted vessels (Figure XG in the [Data Supplement](#)), indicating that miR-128 does not affect this process in vivo. Then, to corroborate the in vitro findings, we measured expression of KLF4 and *Myh11*, observing that miR-128–infused mice had a lower percentage of KLF4-positive cells (Figure 8C) and a concomitant increase in *Myh11* protein distribution (Figure 8D). However, no significant changes in the expression of ACTA2 were observed (Figure XH in the [Data Supplement](#)).

Altogether, these findings clearly show that miR-128 can exert a strong effect also in vivo, modulating VSMC proliferation and migration through direct regulation of KLF4 and expression of *Myh11*.

DISCUSSION

Abnormal proliferation of VSMCs is a major pathogenic cause of cardiovascular diseases, such as atherosclerosis, restenosis, and aneurysm.^{46,47} Of note, current therapeutic approaches for occlusive proliferative vascular pathologies include the use of drug-eluting stents. These devices physically dilate the vascular lumen while releasing an antiproliferative drug inhibiting secondary VSMC reactivation and migration into the lumen.⁴⁸ However, a limitation of antiproliferative drugs is their lack of specificity, as they act equally on all cell types. Cell-specific therapy for arterial restenosis could be reached by taking advantage of miRNAs. The clustered VSMC-specific miR-143 and miR-145 family play a pivotal role in the modulation of VSMC phenotypic switch,^{9,10,12,49} a phenomenon that is controlled at different levels.⁵⁰ Modulation of miR-143 and miR-145 could prevent restenosis,^{12–14,51} but because expression of these 2 miRNAs is very high in vivo, it maybe turn out to be difficult to further increase their expression to a therapeutic level. This may not be the case for miR-128, which is expressed at a level much lower than miR-143/145.

miR-128 is strongly enriched in the brain⁵² and has been studied in central nervous system development.⁵³ Its expression has been negatively associated with the rate of proliferation of glioma cells⁵⁴ and in other cell types, such as cardiomyocytes.²¹ Our findings demonstrate that miR-128 is expressed in VSMCs at a level comparable to that of other contractile cells, including cardiomyocytes and skeletal muscle cells. Similarly to cancer cells,³³ our results indicate that miR-128 strongly controls VSMC proliferation and migration.

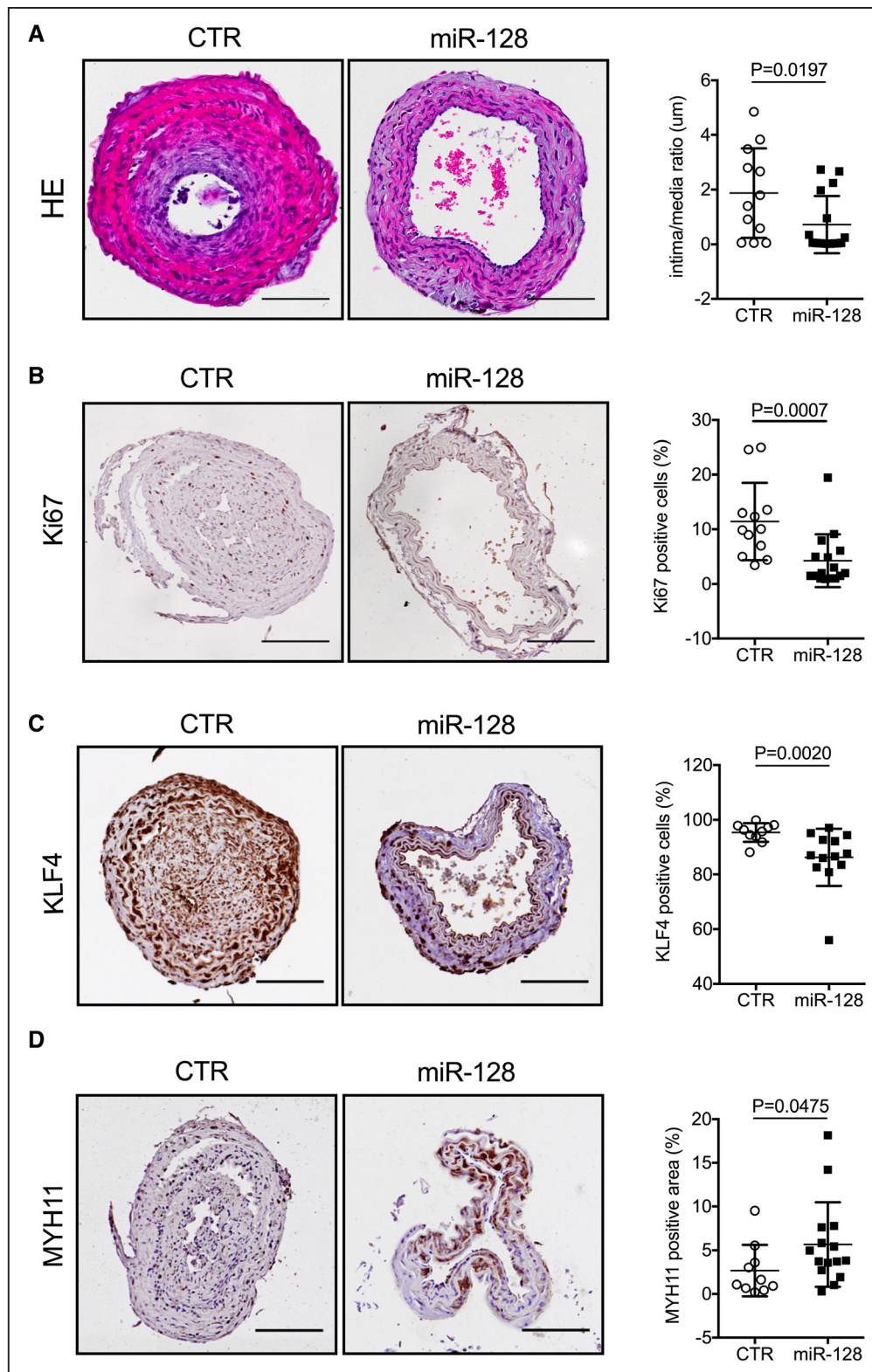


Figure 8. Overexpression of microRNA-128-3p (miR-128) reduces neointima formation in injured carotids.

ApoE^{-/-} mice were infused with control cells (CTR) or miR-128 overexpressing lentiviral particles and then subjected to perivascular collar placement of the left carotid artery. Representative hematoxylin-eosin staining of carotid artery cross-sections and relative quantification of media/intima thickness in both experimental groups (CTR=12, miR-128=16; **A**), Ki-67 staining and proliferative cells quantification (CTR=12, miR-128=16; **B**), KLF4 (Kruppel-like factor 4) staining and positive cells quantification (CTR=10, miR-128=13; **C**), Myh11 (myosin heavy chain 11) staining and positive area quantification (CTR=10, miR-128=15; **D**). Representative images chosen for similarity to the global quantification. Data normality was calculated with Kolmogorov-Smirnov test. Scale bars: 100 μ m. Data represent the mean \pm SD. To compare means, Mann-Whitney test was used.

Another interesting aspect of miR-128's effects on VSMCs is related to an overall change in the epigenome. Among epigenetic alterations, DNA methylation was recently shown by us to critically regulate VSMC proliferation and differentiation.⁴⁰ Modulation of DNA methylation can be a direct response to environmental or pathological insults, and its global level might strongly influence VSMC phenotype.^{23,55} In line with these findings, miR-128-overexpressing cells exhibited altered levels of Dnmts and Tets, an altered status of global DNA methylation, and demethylation of the promoter of a VSMC differentiation hallmark.

Another key finding is miR-128-induced regulation of KLF4. KLF4 is a stem cell pluripotency gene⁵⁶ shown to be modulated by different miRNAs, including the highly VSMC-enriched miRNA miR-145.⁴⁹ KLF4 behaves as a repressor of VSMC differentiation, inhibiting the expression of genes such as *Acta2*, *Sm22*, and *Myh11*.^{57–59} It was also suggested that KLF4 affects DNA methylation.^{41,42} The existence of a link between miR-128, KLF4, and DNA methylation is also supported by identification of unmethylated CpG islands in the *Myh11* promoter of miR-128-overexpressing VSMCs. Thus, considering also the finding that forced expression of miR-128 in non-VSMC types triggers a VSMC-like phenotype, it is possible that modulation of miR-128 might affect stemness. Nonetheless, we can also speculate that miR-128 might affect DNA methylation through a KLF4-independent mechanism, as it could directly regulate other genes, such as *Suz12*.²¹

In summary, our study demonstrates that VSMC phenotype can be modulated in vitro and in vivo by altering the expression of miR-128. A therapeutic application of this miRNA for cardiovascular diseases characterized by VSMC proliferation could be foreseen.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Materials

Expanded Materials & Methods

Online Figures I–X

Online Table I–III

Data Sets (Supplemental file I, II, and III)

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