YMTHE, Volume *33*

Supplemental Information

Functional screening identifies miRNAs with a novel function inhibiting vascular smooth muscle cell proliferation

Julie Rodor, Eftychia Klimi, Simon D. Brown, Georgios Krilis, Luca Braga, Nadja A.R. Ring, Margaret D. Ballantyne, Despoina Kesidou, Aurelie Nguyen Dinh Cat, Vladislav Miscianinov, Francesca Vacante, Katarina Miteva, Matthew Bennett, Abdelaziz Beqqali, Mauro Giacca, Serena Zacchigna, and Andrew H. Baker

Supplemental methods

Caspase-3 assay for apoptosis analysis in HSVSMCs

Caspase-3 activity in HSVSMC lysates was quantified colorimetrically using the Caspase-3 Assay Kit from Abcam (ab39401), according to the manufacturer's protocol. Measurement was done on cell lysates with the same total protein concentration. Absorbance at 405 nm was measured using a Molecular Devices microplate reader. Cells treated with MG-115 at 1 µM concentration were used as a positive control.

SMC-condition medium stimulation of HSVECs

Human saphenous vein smooth muscle cells conditioned media (CM-SMC) from each non transfected and transfected experimental conditions including transfection with each 7 miRNA mimics were collected, centrifuged at 1000 g for 10 min at 4ºC and sterile-filtered with 0.2 µm filters (Millipore). In 6 well-plates, 12 hours-quiescent HSVECs were stimulated with 500 mL of CM-SMC were added to 500 mL of EC basal medium with 10 µM EdU for 48 hours.

Proliferation was assessed using the Click-it EdU (5-Ethynyl-2´-deoxyuridine) 488 Proliferation assay (Life Technologies, UK) as per manufacturer's instructions. Cells were then dissociated and fixed in ice cold 70 % ethanol for EdU flow cytometry analysis. EdU incorporation was confirmed using anti-EdU 488 antibody.

Network analysis and visualisation

Genes commonly regulated by all miRNAs and candidate targets of each miRNA were considered. Genes involved in cell cycle regulation were extracted based on the GO Term annotation GO:0007049. Network analysis was done on these 176 genes. Gene interactions were obtained using STRING version 12.0 (https://string-db.org/) with default parameters. Visualisation of the network was performed using Cytoscape version 3.9.1. Genes commonly regulated by all miRNAs were placed at the centre of the network while targets were placed at the periphery.

Supplemental Tables

Table S1: miRNA screen result in HPASMC (Excel file).

Table S2: Differentially expressed genes between each miRNA mimic condition versus miR-CTRL in HSVSMC (Excel file).

Table S3: Gene set enrichment analysis (Biological Process GO Terms) for the differentially expressed genes between each miRNA mimic condition versus miR-CTRL in HSVSMC (Excel file).

Table S4: Candidate target genes for each candidate miRNA based on their downregulation in the HSVSMC RNAseq and their prediction by at least two prediction tools (Excel file).

Table S5: Differentially expressed genes between each miRNA mimic condition versus miR-CTRL in HSVEC (Excel file).

Table S6: TaqMan probes used for qRT-PCR analysis

Species	miRNA ID	TaqMan Assay ID
Human	hsa-miR-323-3p	002227
Human	hsa-miR-1827	002814
Human	hsa-miR-5681b	476577_mat
Human	hsa-miR-4774-3p	462836_mat
Human	hsa-miR-491-3p	002360
Human	hsa-miR-449b	001608
Human	hsa-miR-892b	002214
Human	RNU48	001006
Human	MKI67	Hs04260396_g1
Human	CCND1	Hs00765553_m1
Human	GJA1	Hs00748445_s1
Human	IGF2BP3	Hs00559907_g1
Human	UBC	Hs01871556_s1

Supplemental Figures

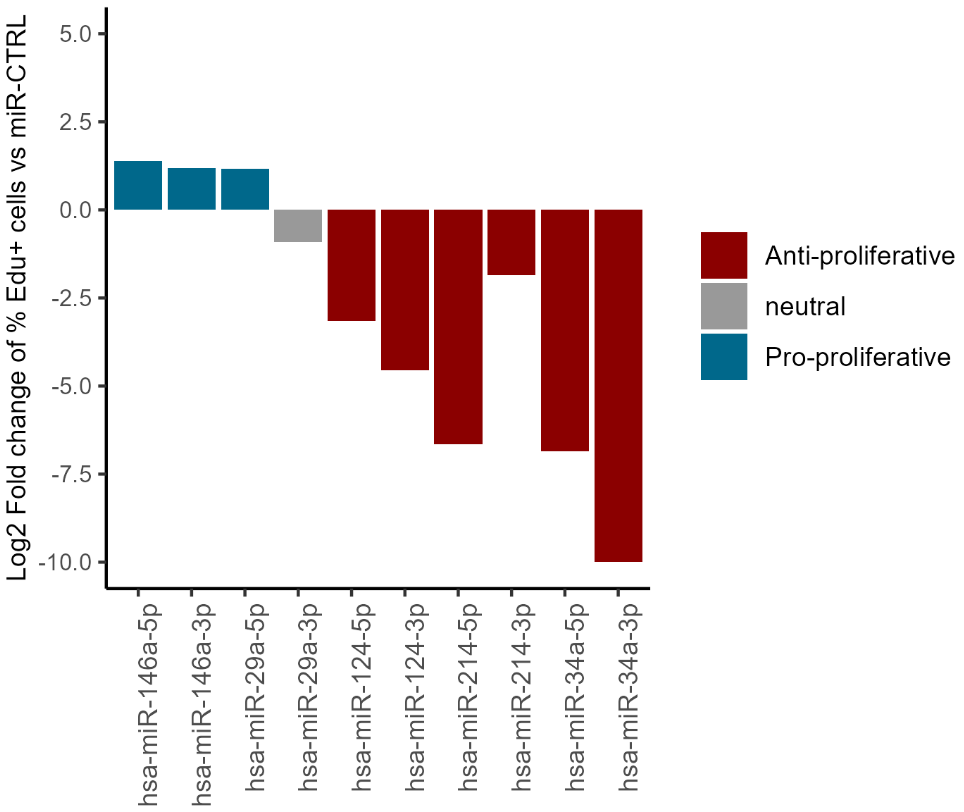


Figure S1. miRNAs with known effect on SMC proliferation (pro-proliferative: miR-146a and miR-29a, and anti-proliferative: miR-124, miR-214 and miR-34a) showed expected changes in the high-throughput high content miRNA screening in human pulmonary artery SMCs (HPASMC).

Bar graph showing the changes in the percentage (%) of Edu-positive cells (Edu+) after mimic-based overexpression of miRNAs vs miR-CTRL. As the log fold change for miR-34a-3p overexpression was – infinite; we used an arbitrary value of -10 for plotting.

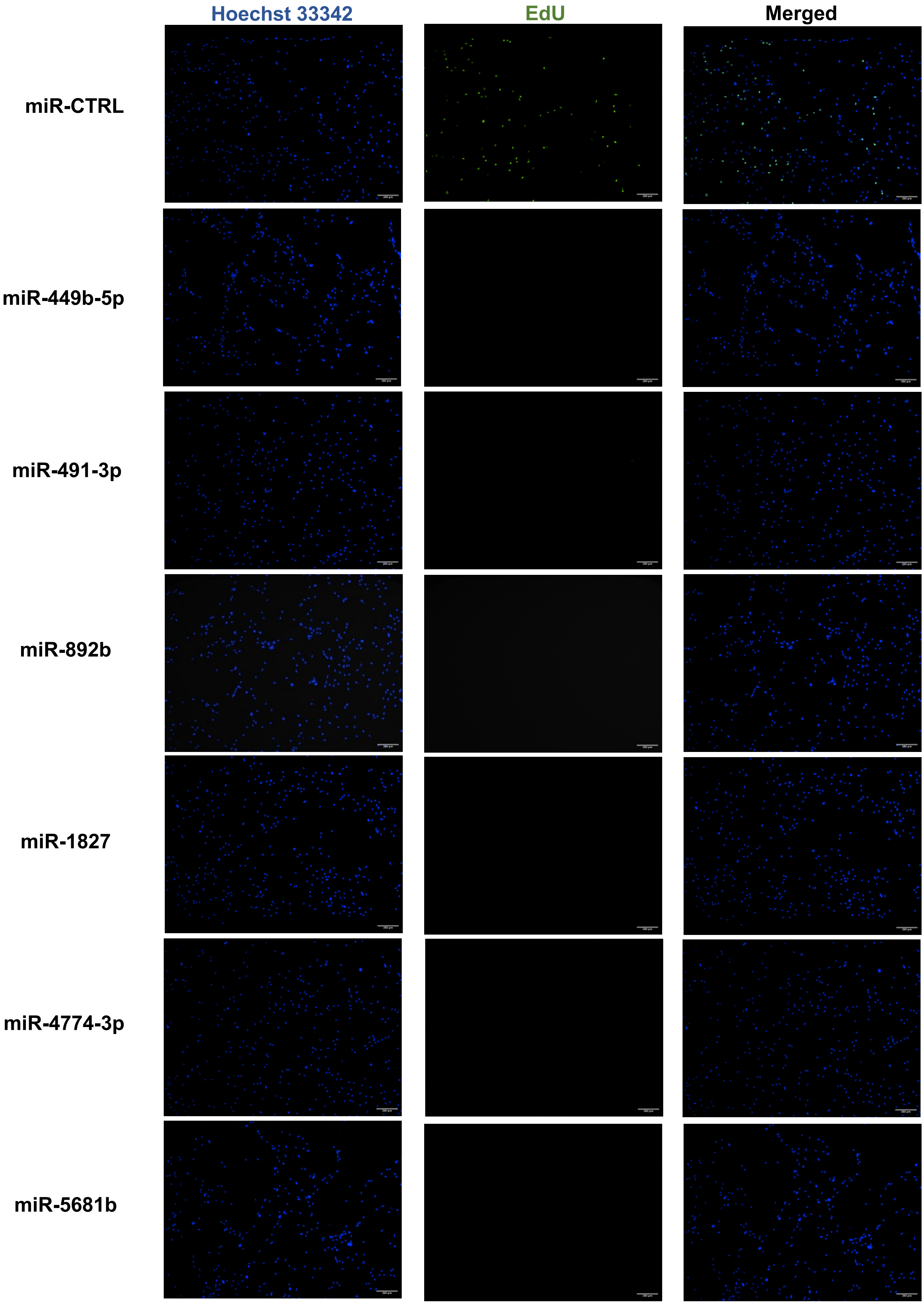


Figure S2. Representative images of human HPASMCs stained with Hoechst 33342 (blue) and EdU (green) following treatment with miR-CTRL or mimics for miR-449b-5p, miR-491-3p, miR-892b, miR-1827, miR-4774-3p and miR-5681b. Scale bar is 100μm.

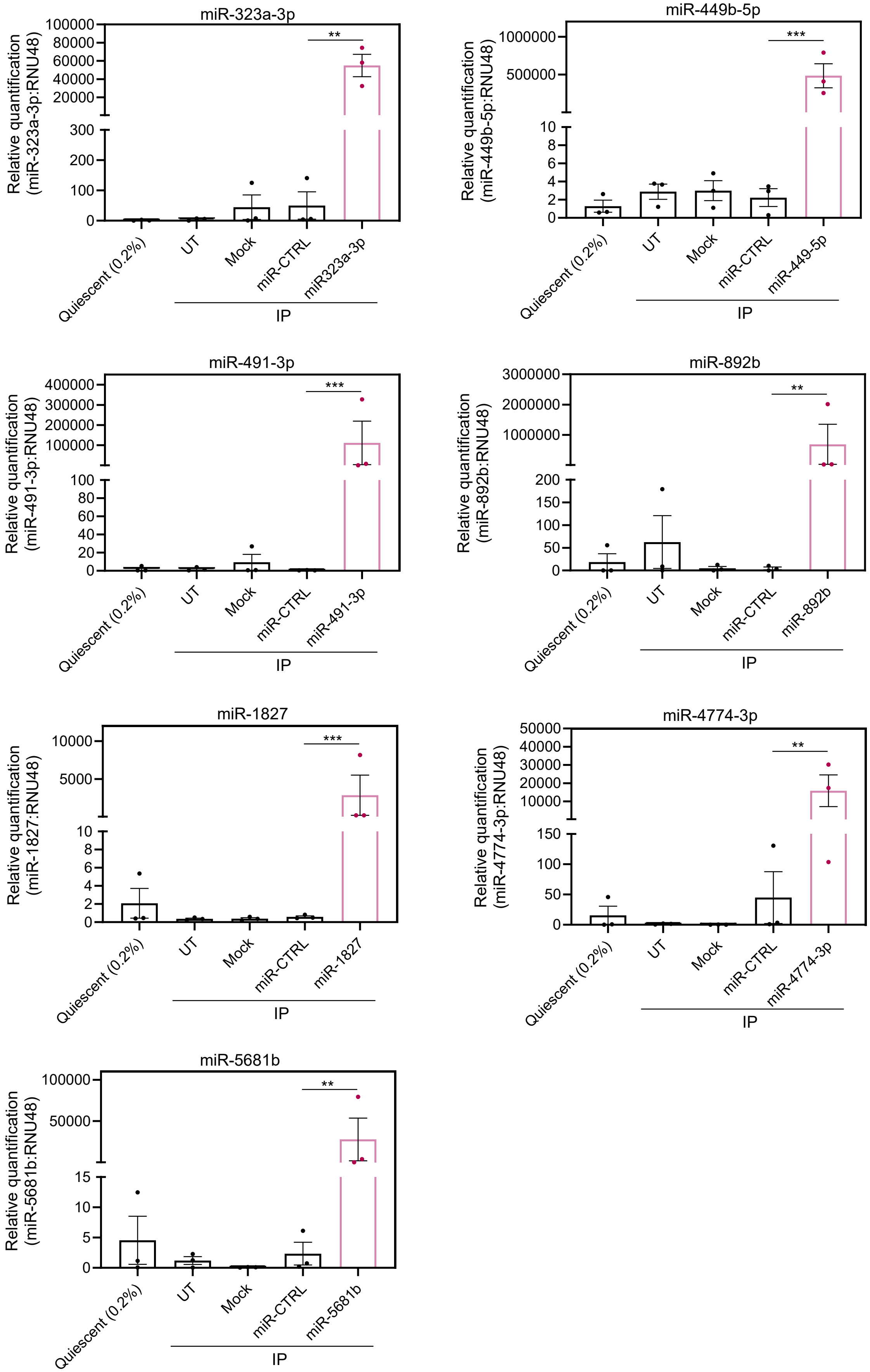


Figure S3: Confirmation of miRNA overexpression using mimics for miRNA miR-323a-3p, miR-449b-5p, miR-491-3p, miR-892b, miR-1827, miR-4774-3p, miR5681b in HSVSMC by RT-qPCR (n=3). UT: Untransfected, IP: IL-1α+PDGF-BB. Mock corresponds to lipofectamine only.

Statistical analysis was performed using a repeated measures ANOVA. P-values for the comparison between miRNA mimic treatment and miR-CTRL treatment obtained after Dunnet's test for multiple corrections are included on the graphs: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. n numbers correspond to distinct biological replicates.

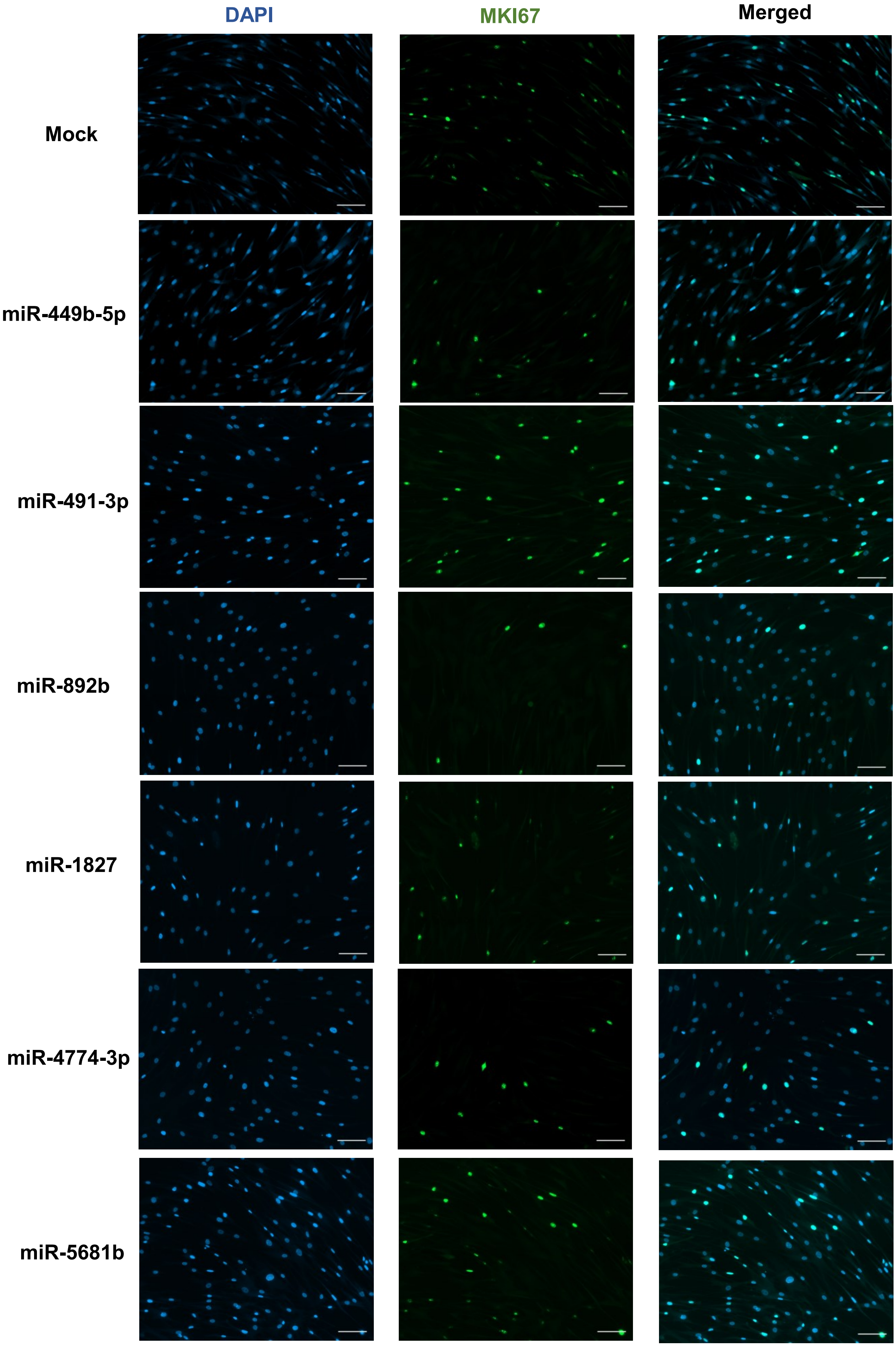


Figure S4: Representative images of MKI67 and DAPI staining of IL-1α/PDGF-BB-stimulated HSVSMCs transfected with mimic for miR-449b-5p, miR-491-3p, miR-892b, miR-1827, miR-4774-3p, miR5681b or Mock condition (lipofectamine only). Scale bar is 100μm.

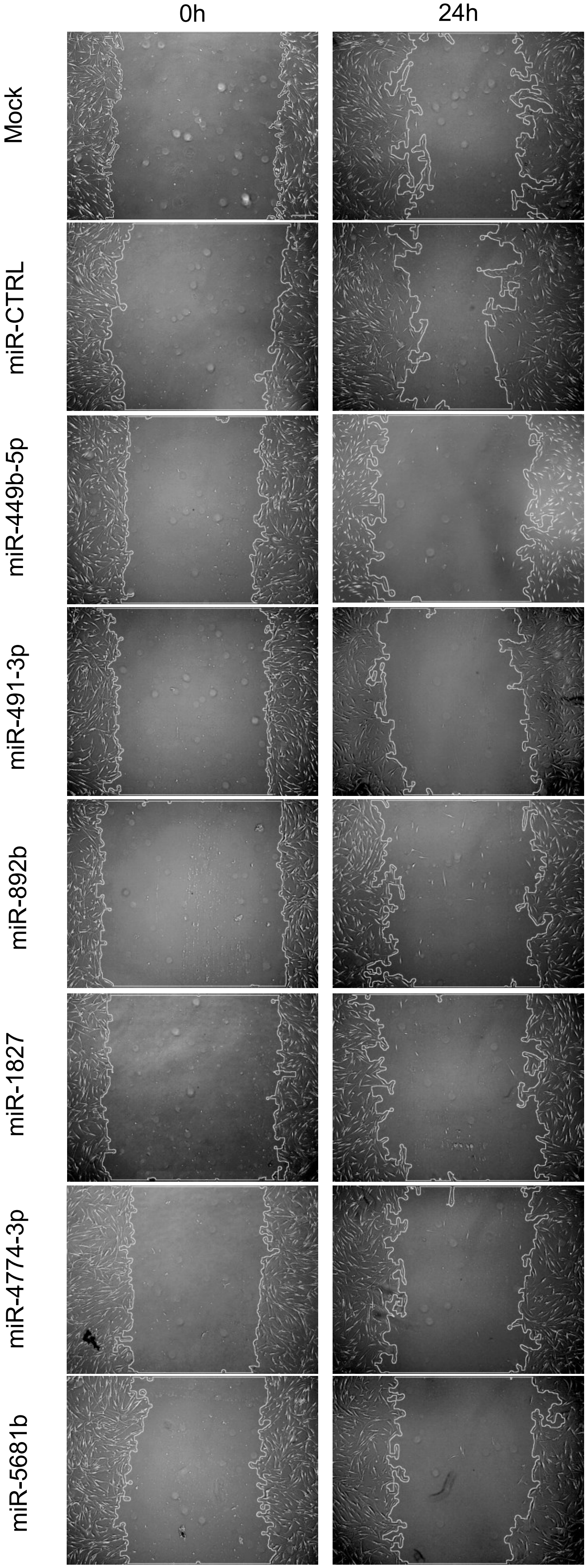


Figure S5: Wound healing assay of IL-1α/PDGF-BB-stimulated HSVSMCs transfected with the 7 miRNA mimics or miR-CTRL. Representative images from scratch assay at 0 and 24hours for miR-CTRL, miR-449b-5p, miR-491-3p, miR-892b, miR-1827, miR-4774-3p or miR-5681b. Scale bar is 500μm.

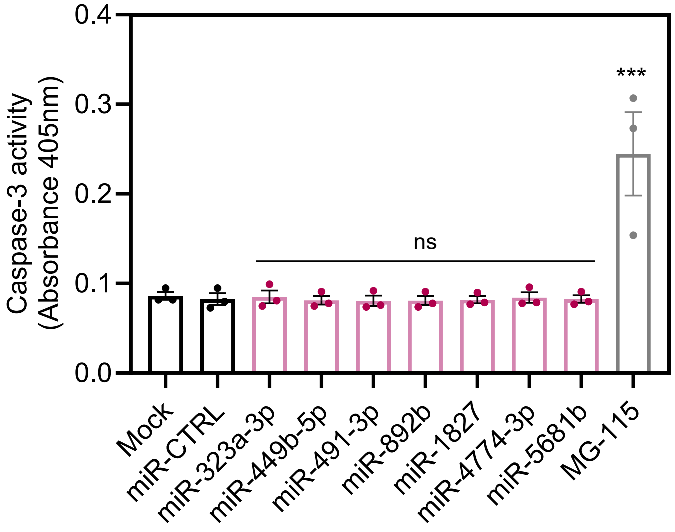
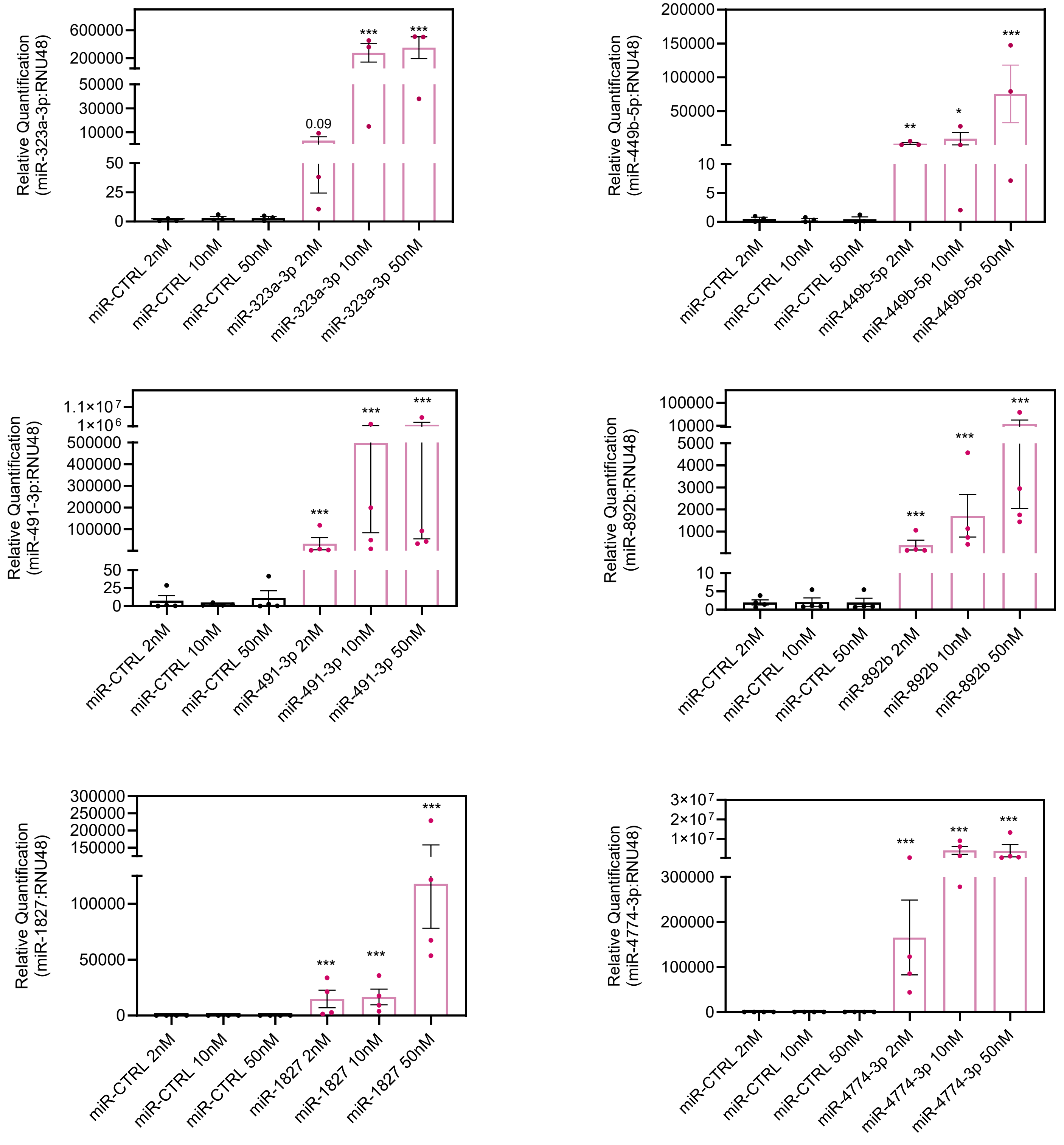


Figure S6: Quantification of Caspase-3 activity (absorbance measured at 405nm) in IL-1α/PDGF-BB -stimulated HSVSMCs transfected with the 7 miRNA mimics or miR-CTRL, as well as the "mock" transfection control (n=3). The proteasome inhibitor MG-115 was used as a positive control for apoptosis induction. Statistical analyses were done using Iman-Conover non-parametric ranking followed by repeated measures ANOVA. P-values for the comparison between miRNA mimic treatment and miR-CTRL treatment obtained after Dunnet's test for multiple corrections are included on the graph: ***$P < 0.001$, ns= non-significant. n numbers correspond to distinct biological replicates.



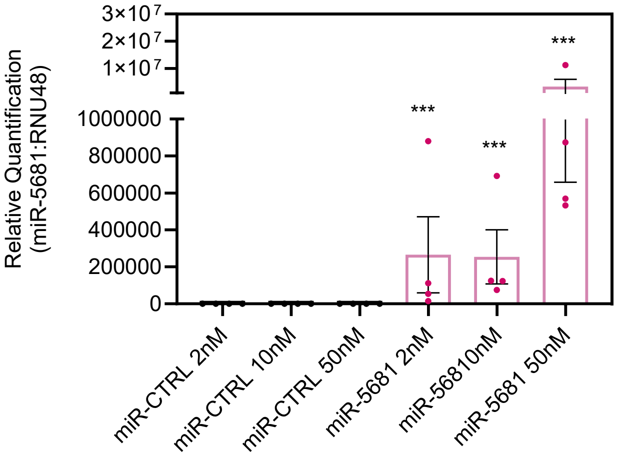


Figure S7: miRNA expression level detected by RT-qPCR in a dose response experiment using mimics concentration of 2, 10 and 50nM in HSVSMC treated with IL-1α and PDGF-BB (n=3-4). Statistical analysis was performed using a repeated measures ANOVA. P-values for the comparison between miRNA mimic treatment and their respective miR-CTRL treatment at the same concentration obtained after Tukey's test for multiple corrections are included on the graphs: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n numbers correspond to distinct biological replicates.

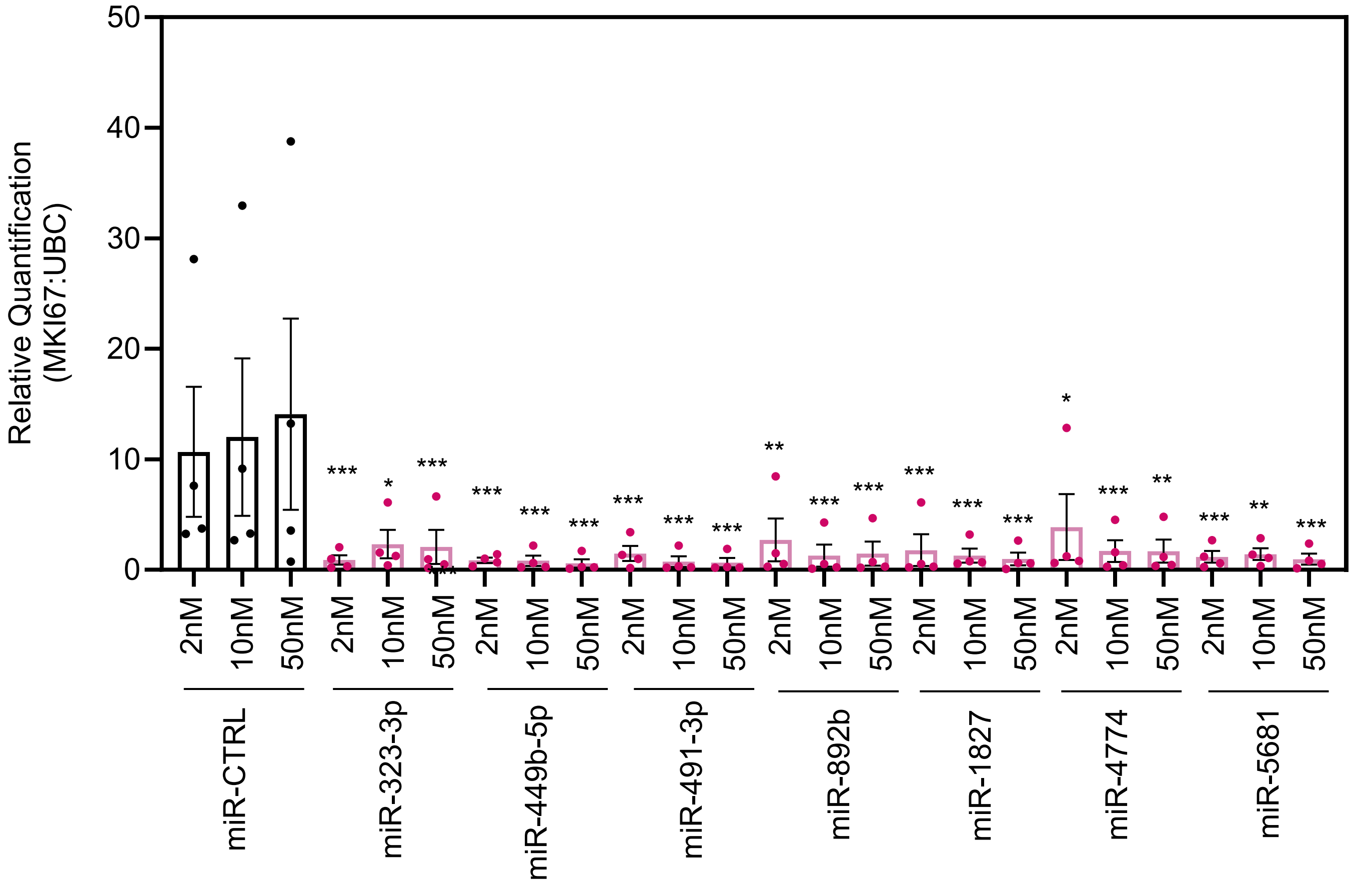


Figure S8: Effect of different doses of miRNA mimics on proliferation based on level of expression of MKI67 by RT-qPCR. HSVSMC were transfected with 3 different doses (2, 10 and 50nM) of mimics of the candidate miRNAs then treated with IL-1α and PDGF-BB (n=4). Statistical analysis was performed using a repeated measures ANOVA. P-values for the comparison between miRNA mimic treatment and their respective miR-CTRL treatment at the same concentration obtained after Tukey's test for multiple corrections are included on the graph: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. n numbers correspond to distinct biological replicates.

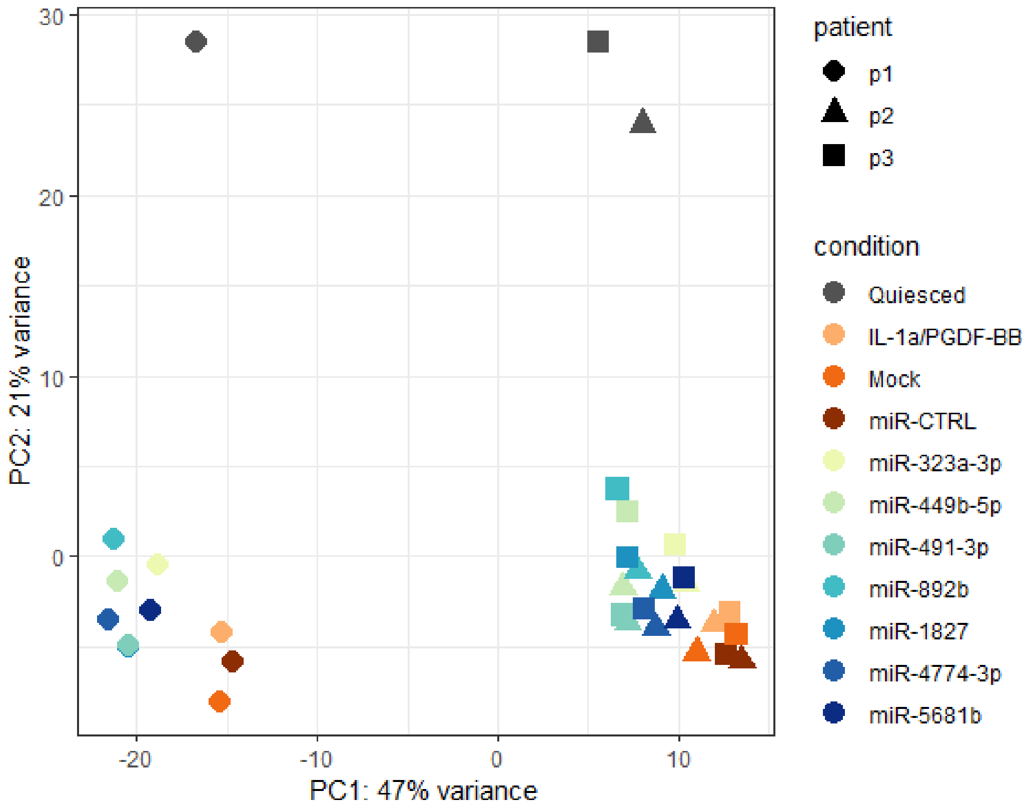


Figure S9: Principal component analysis plots of the HSVSMC RNAseq without removal of batch effect (removal of patient effect).

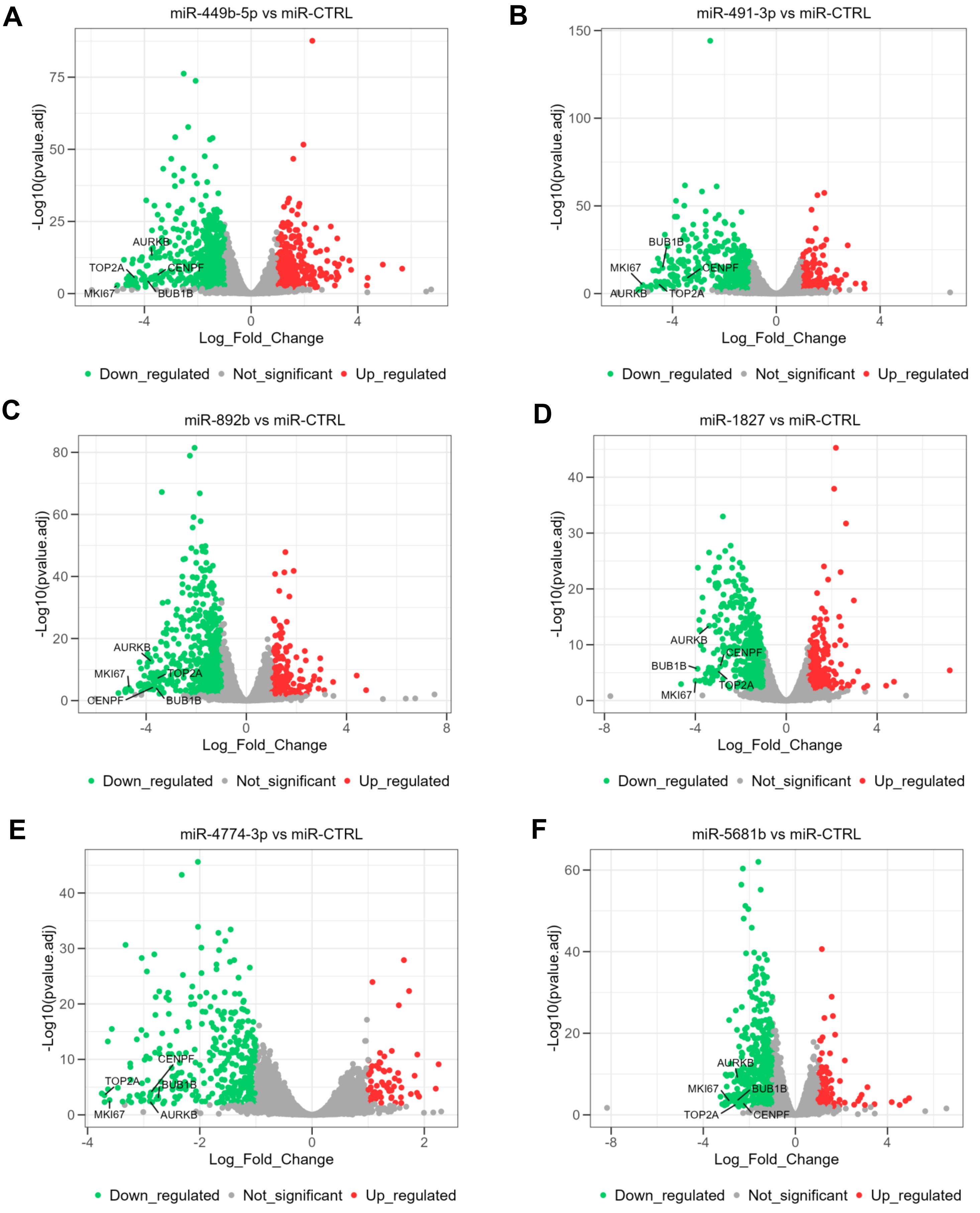


Figure S10: Volcano plot of the differentially expressed genes upon each miRNA overexpression (A) miR-449b-5p, (B) miR-491-3p, (C) miR-892b, (D) miR-1827, (E) miR-4774-3p and (F) miR-5681b . The comparisons were performed using DESeq2. Significant changes were identified using a threshold of absolute Fold Change >=2 and adjusted p value<0.01. Cell cycle genes MKI67, TOP2A, BUB1B, AURKB and CENPF are highlighted.

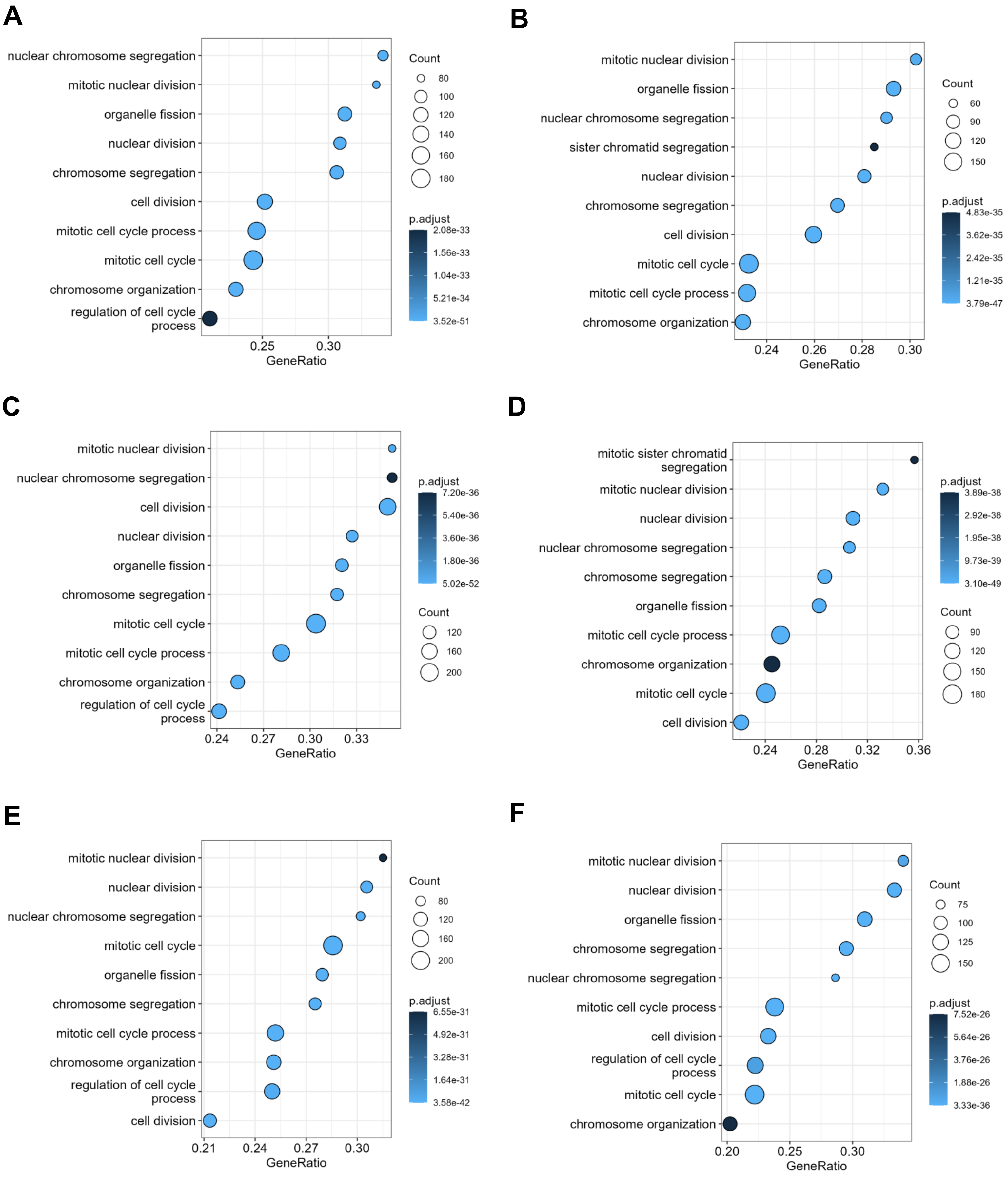


Figure S11: Top10 enriched Go Terms (Biological Process) based on gene set enrichment analysis for the genes differentially expressed upon (A) miR-449b-5p, (B) miR-491-3p, (C) miR-892b, (D) miR-1827, (E) miR-4774-3p and (F) miR-5681b overexpression.

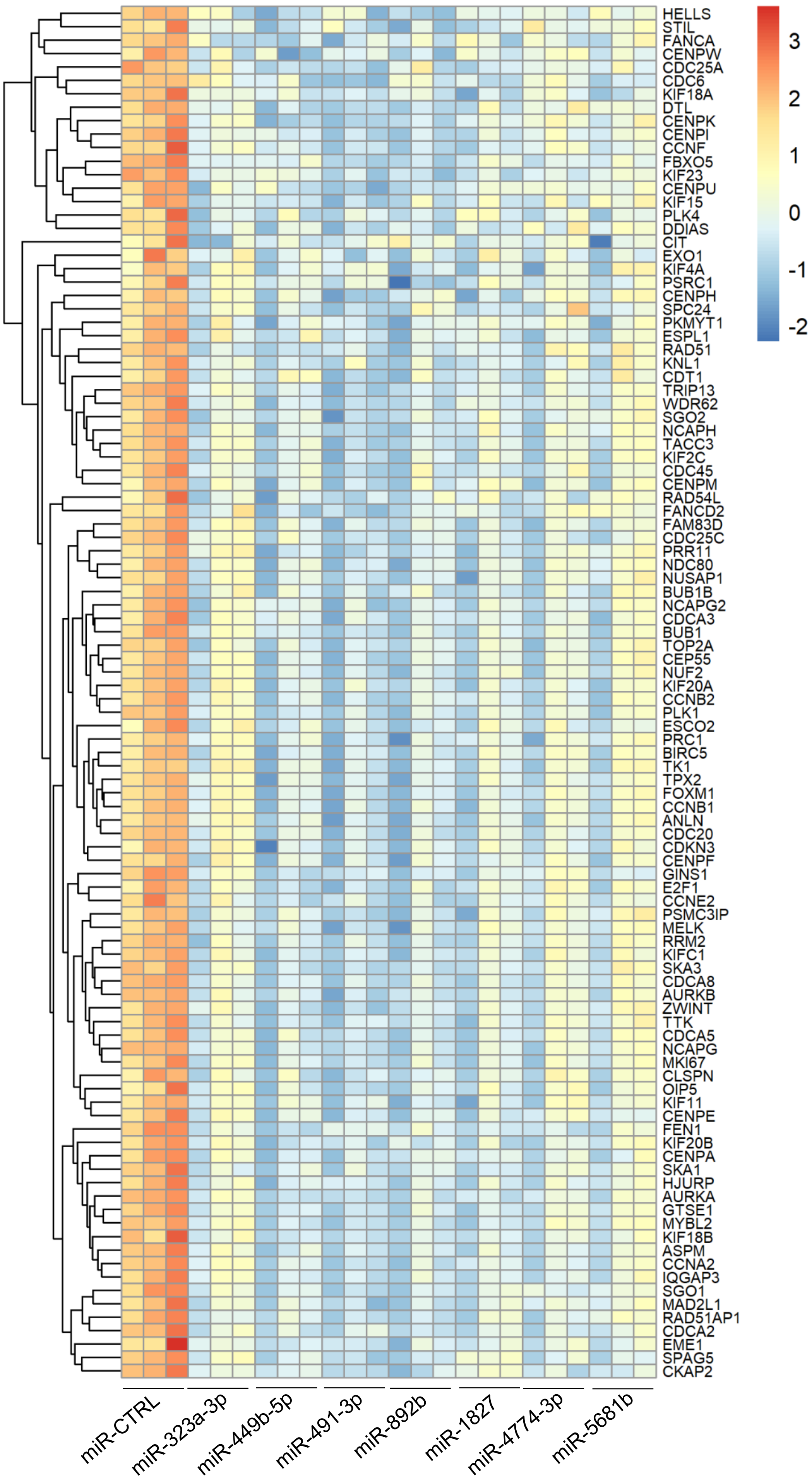


Figure S12: Heatmap of the 102 cell-cycle genes commonly down-regulated by all 7 mimics miRNAs in HSVSMC. Expression displayed as row z-score of Log2(FPKM+1)

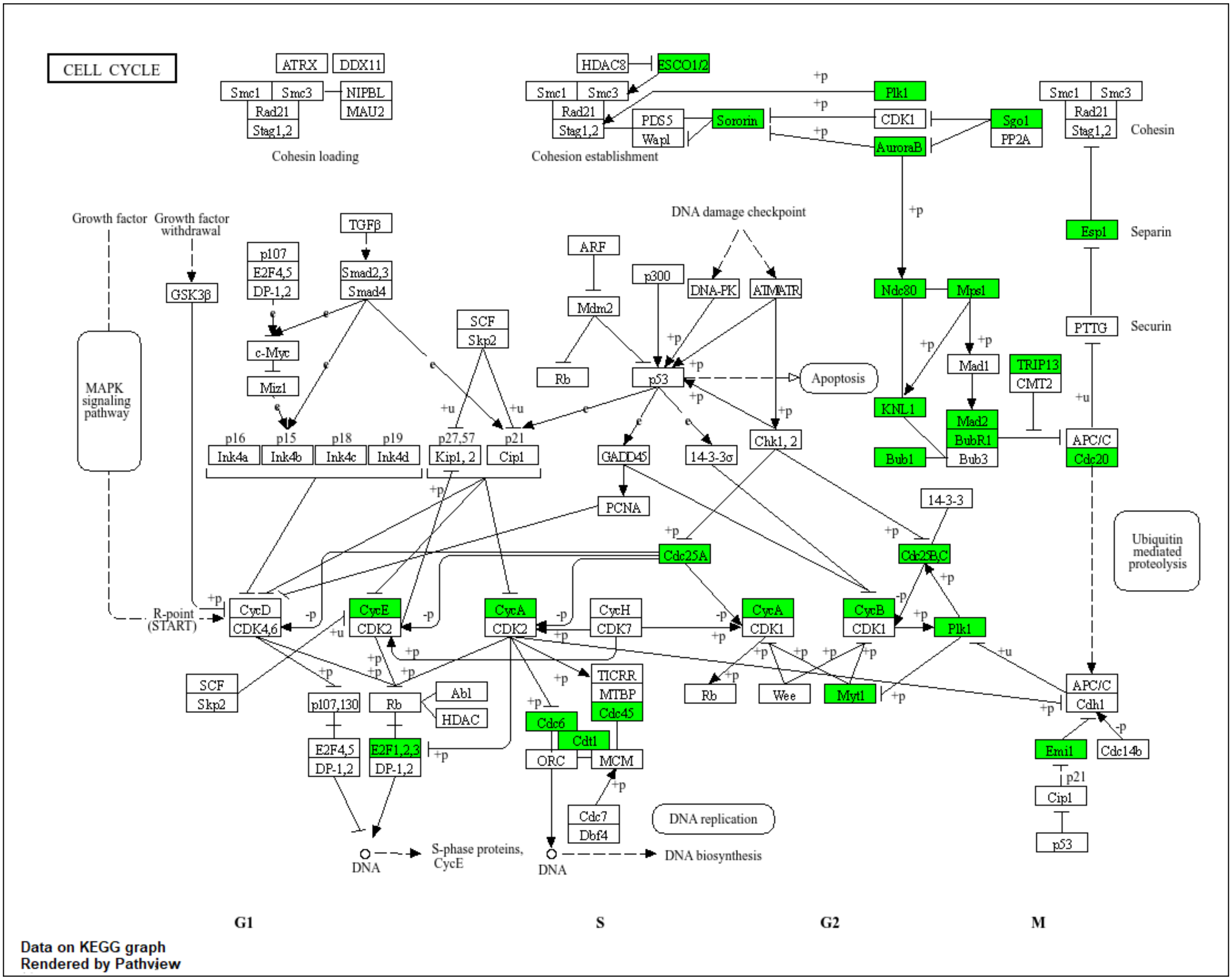


Figure S13: Diagram showing the genes regulated by all 7 miRNAs (in green) involved in cell cycle KEGG pathway (hsa04110) generated using Pathview.

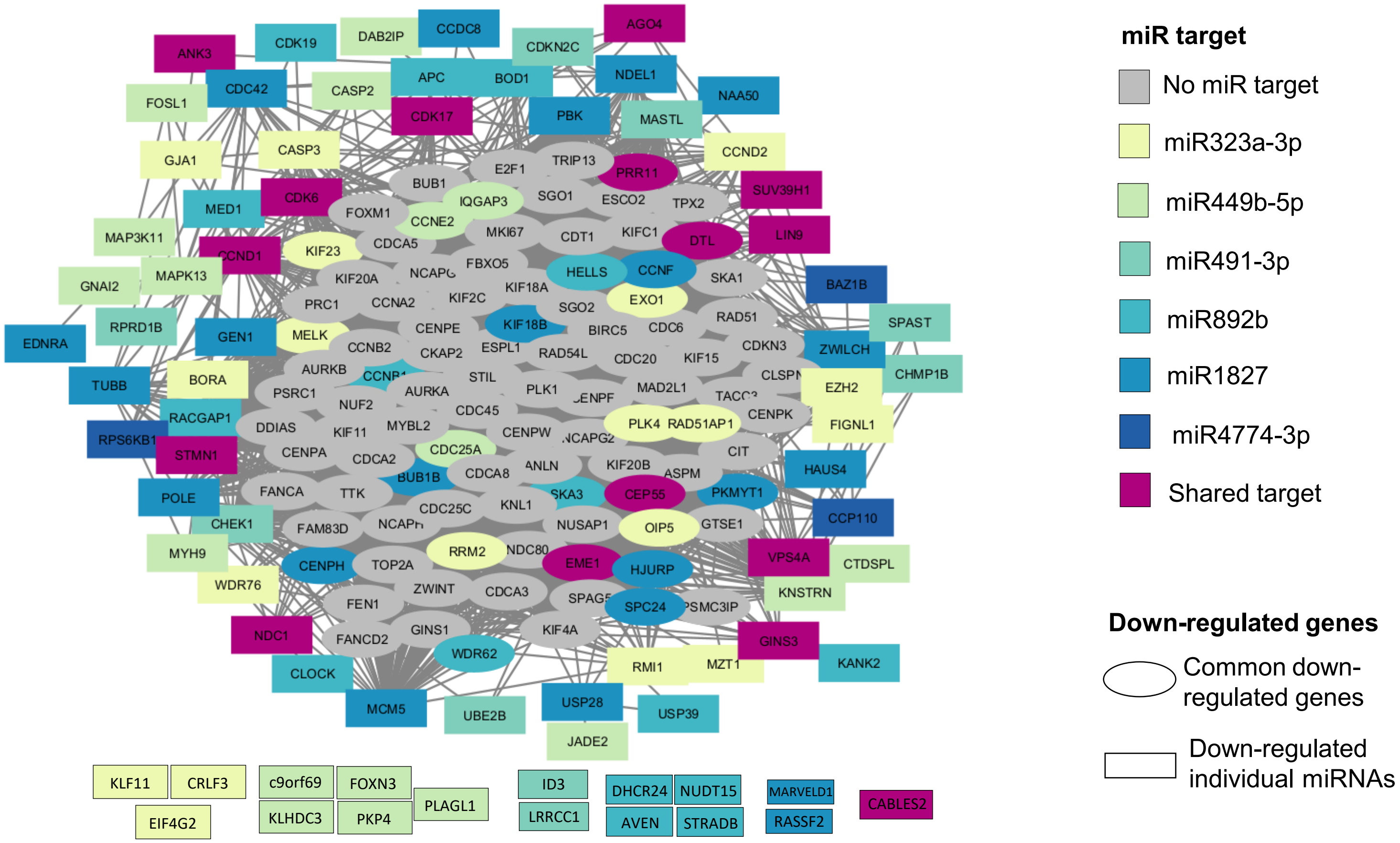


Figure S14: Network of candidate target genes and commonly down-regulated genes involved in Cell Cycle. The predicted gene interactions were obtained using STRING. The network was visualised using Cytoscape. The commonly down-regulated genes are represented in the centre of the network while the targets are located at the periphery.

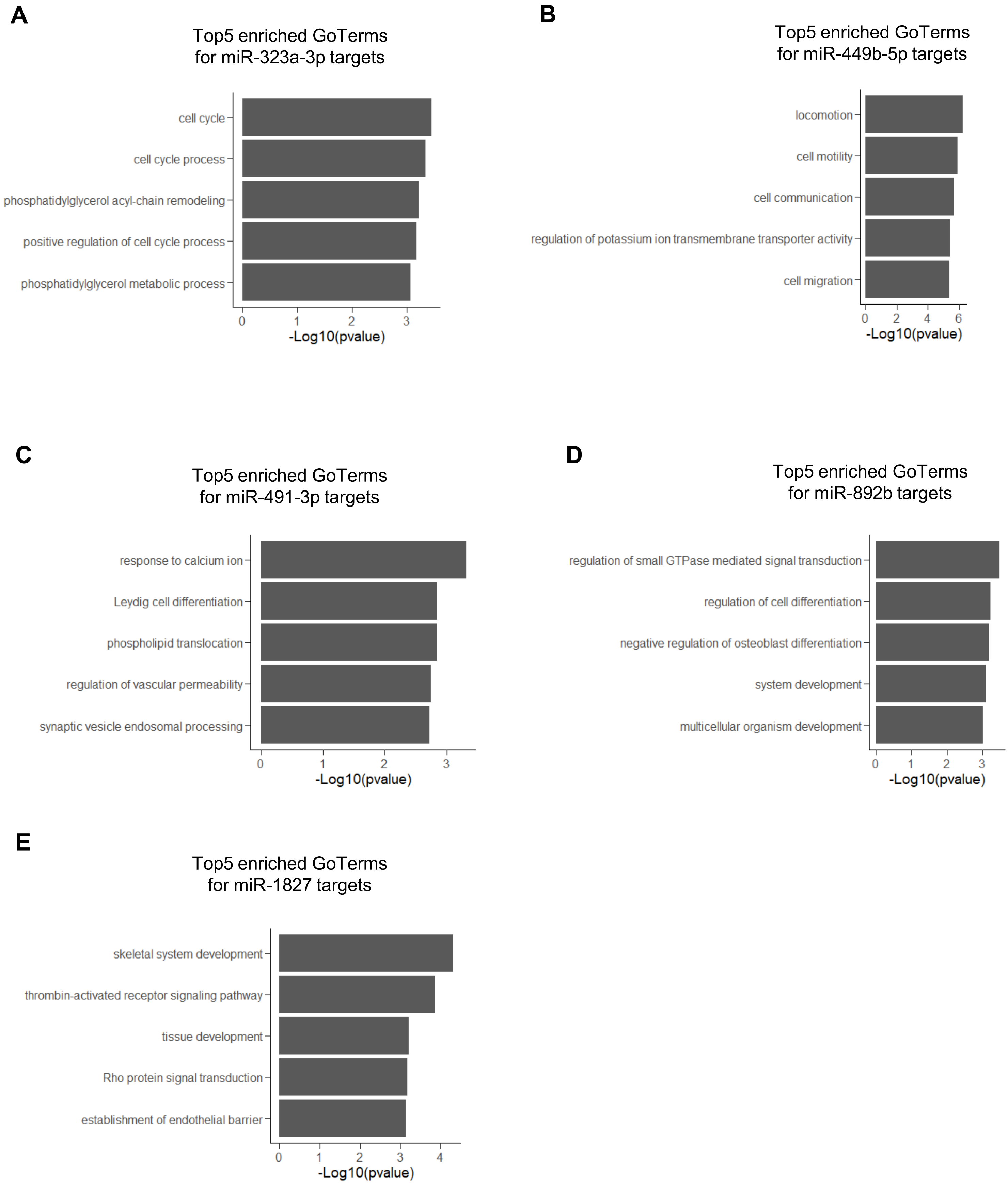


Figure S15: Top5 enriched GoTerns (Biological process) of miRNA candidate targets for (A) miR-323a-3p, (B) miR-449b-5p, (C) miR-491-3p, (D) miR-892b, (E) miR-1827.

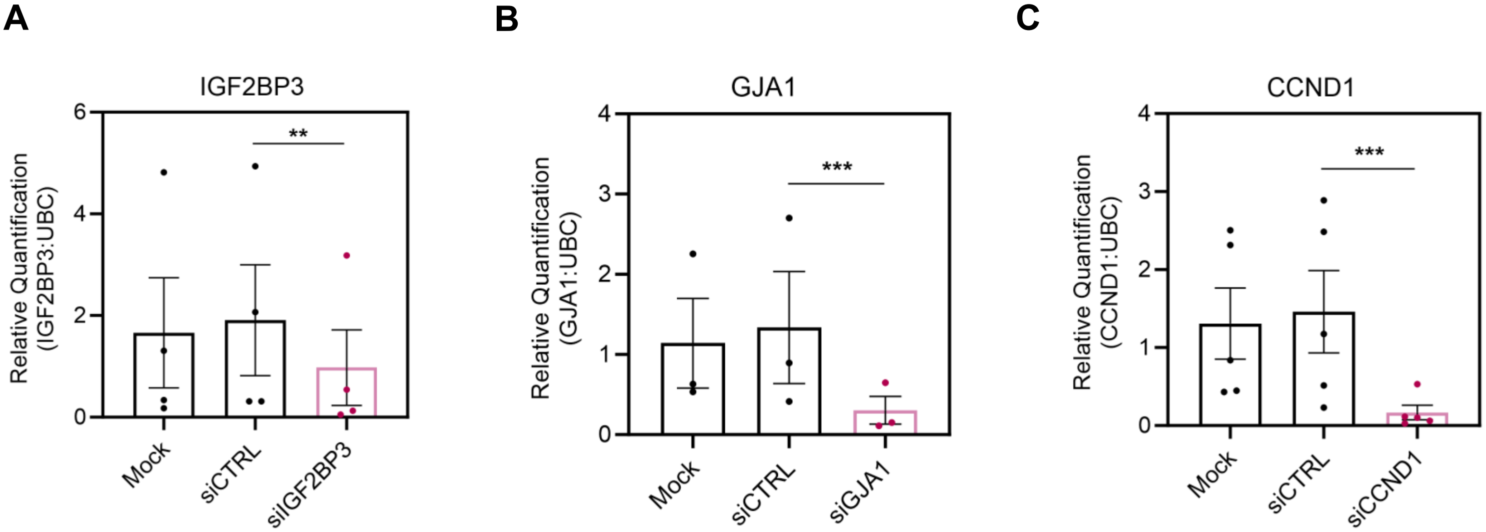


Figure S16: Knockdown validation of miRNA candidate target (A) IGF2BP3 (n=4), (B) GJA1 (n=3) and (C) CCND1 (n=5) in HSVSMC. Expression level was quantified by RT-qPCR after transfection with respective siRNA. Mock corresponds to Lipofectamine only.

Statistical analysis was performed using a repeated measures ANOVA. P-values for the comparison between siRNA target treatment and siRNA Control (siCTRL) obtained after Dunnet's test for multiple corrections are included on the graphs: ***P* < 0.01, ****P* < 0.001. n numbers correspond to distinct biological replicates.

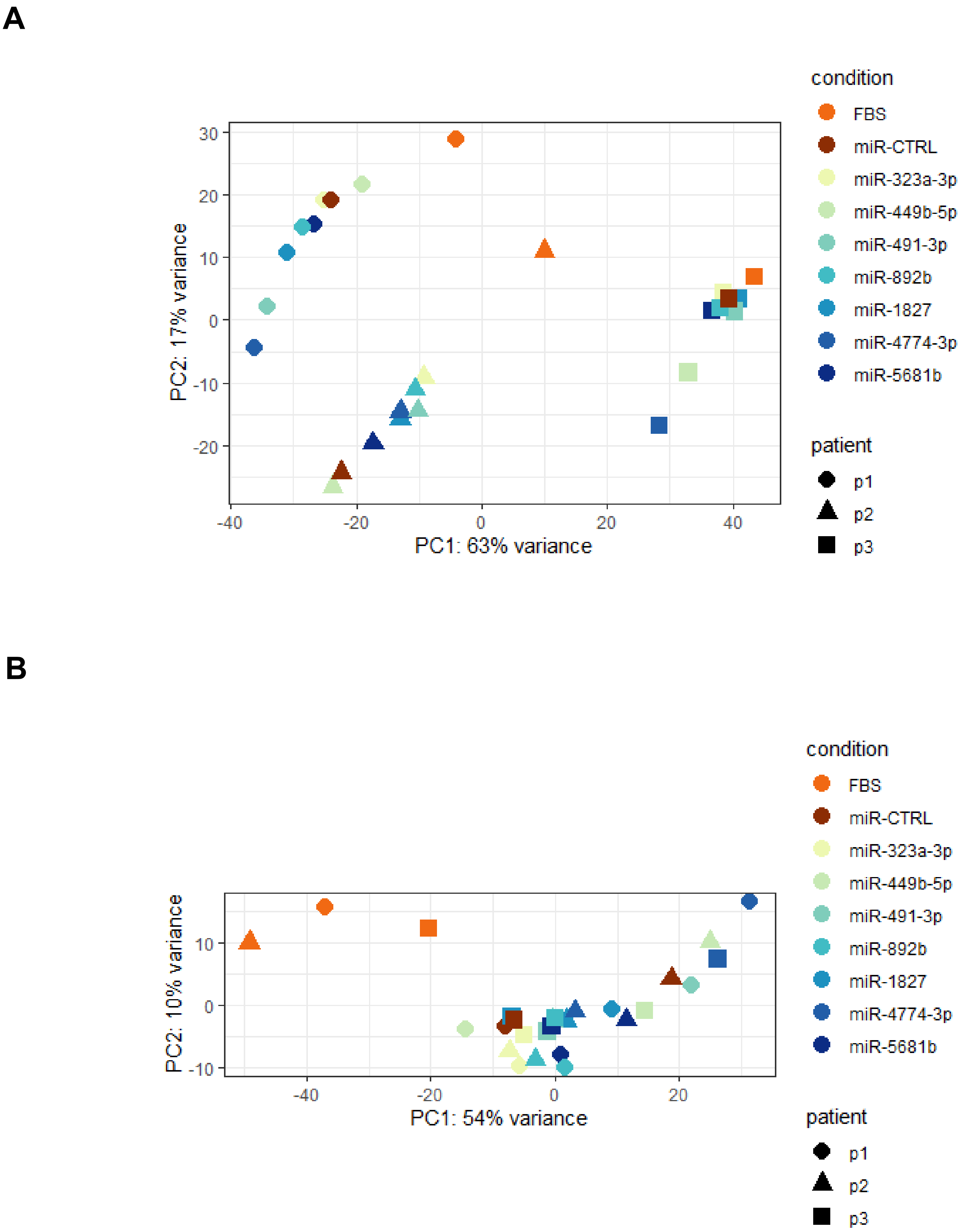


Figure S17: Principal component analysis plot of the HSVEC RNAseq without (A) and with (B) removal batch effect (removal of patient effect).

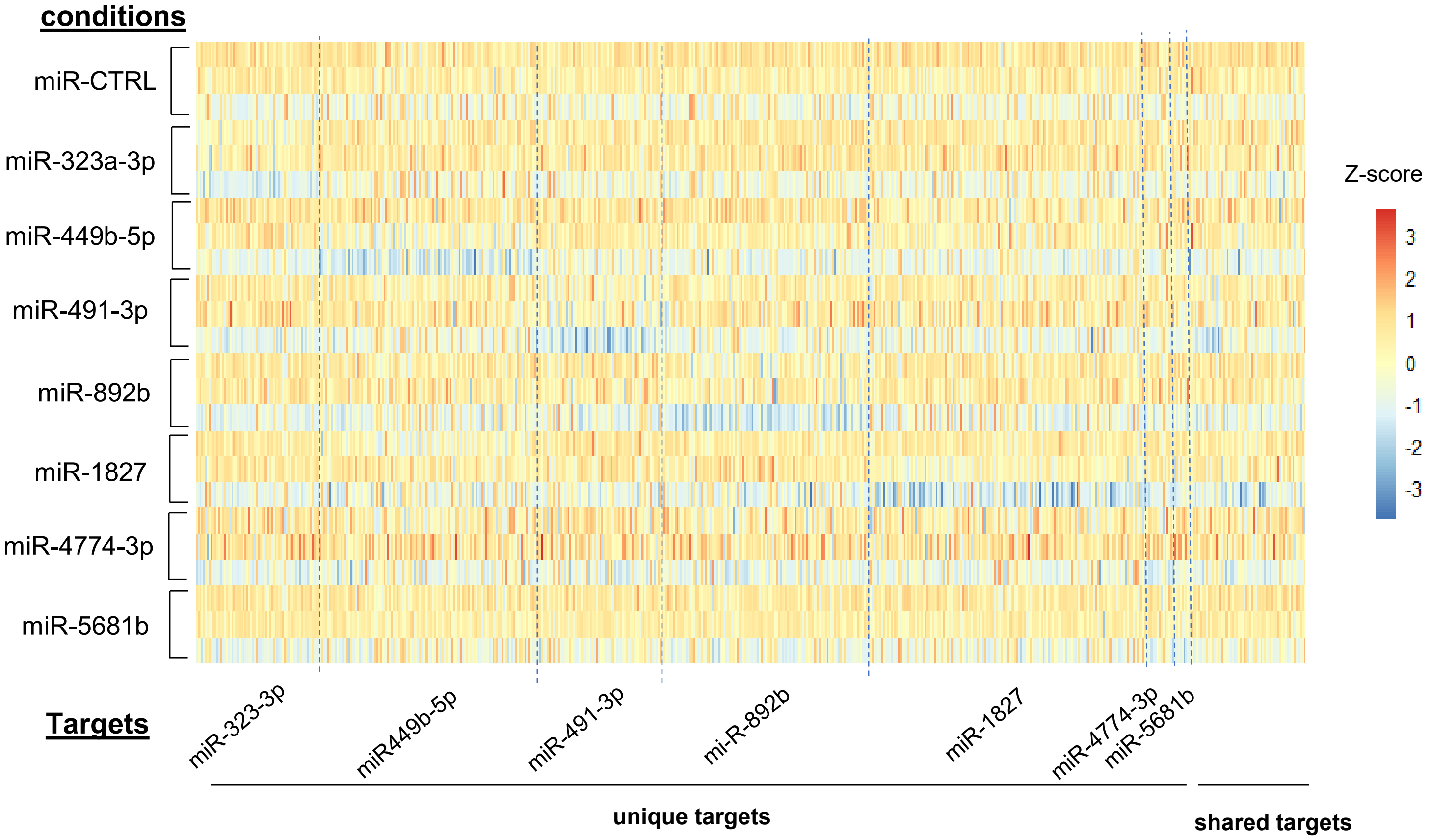


Figure S18: Heatmap showing the expression profile in HSVEC of all miRNA candidate targets identified in the HSVSMC RNAseq with a separation between unique and shared targets. Expression displayed as column z-score of Log2(FPKM+1).

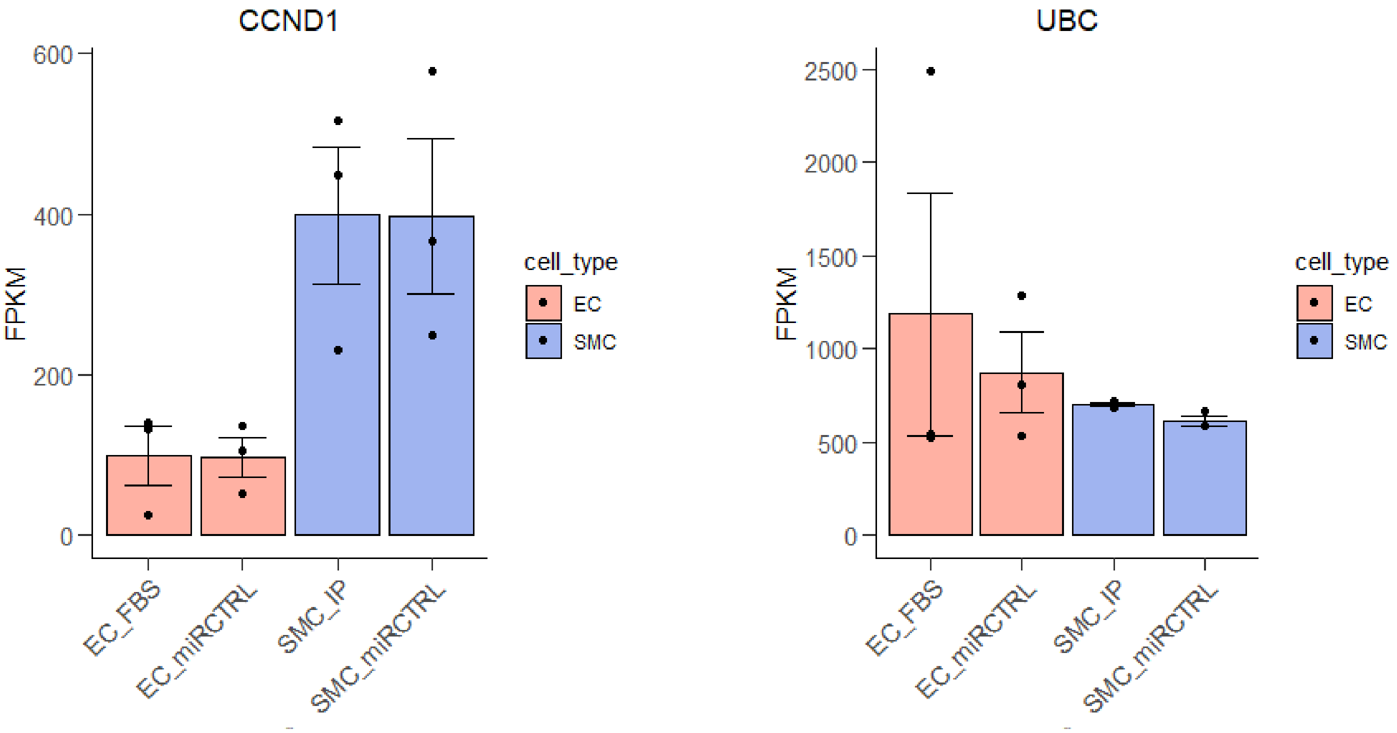


Figure S19: Expression level (as FPKM based on RNAseq) of CCND1 and UBC in HSVEC and HSVSMC under proliferative condition (FBS/IP) or with miR-CTRL treatment. IP: IL-1α+PDGF-BB treatment.

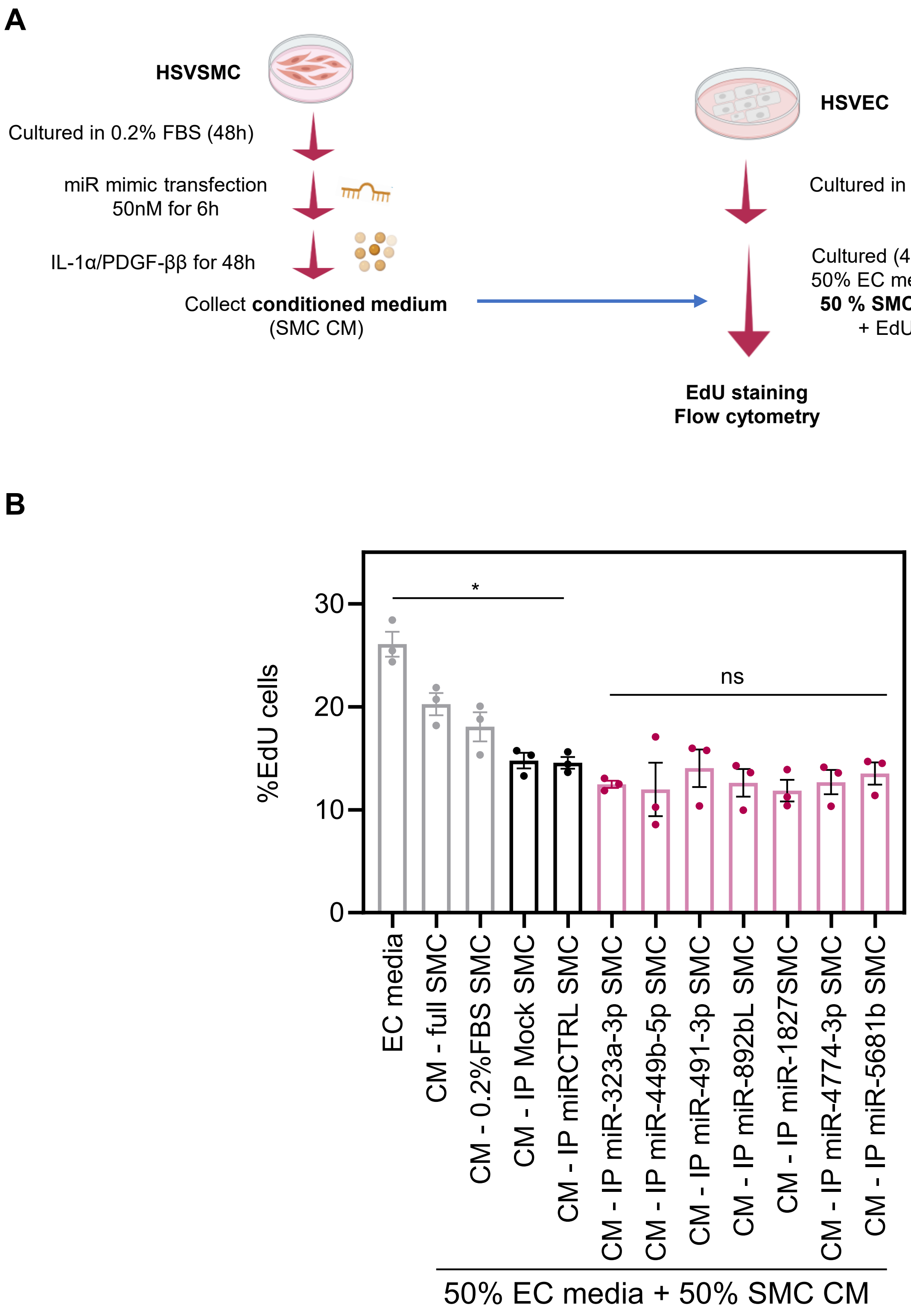


Figure S20: The overexpression of the candidate miRNA in HSVSMC does not lead to a negative paracrine effect on HSVEC proliferation *in-vitro*. (A) Schematic of the collection of conditioned media from HSVSMC treated with miR mimics and the treatment of HSVEC with the conditioned media. (B) Percentage of EdU+ HSVEC cells cultured with EC media or conditioned media from HSVSMC treated with miR-CTRL or indicated miR mimics.
IP: IL-1α+PDGF-BB. Mock: lipofectamine treated cells.

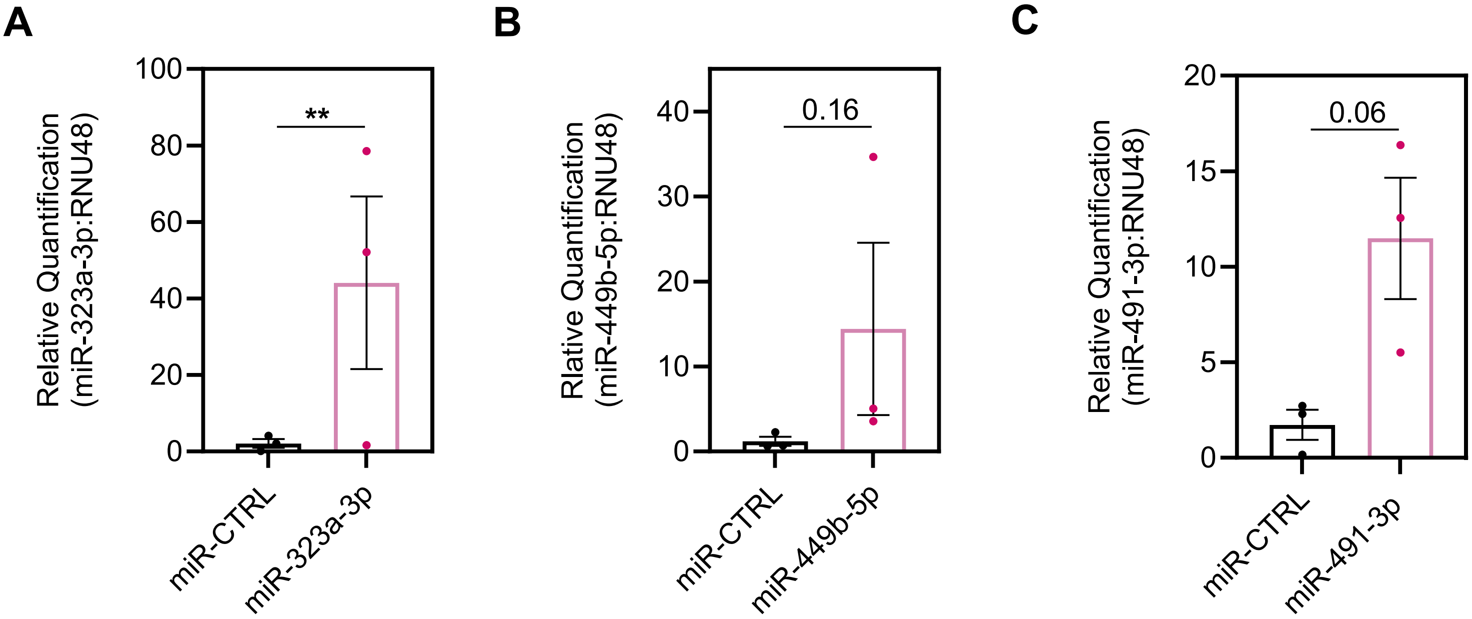


Figure S21: Quantification of (A) miR-323a-3p, (B) miR-449b-5p and (C) miR-491-3p expression level by RT-qPCR after transfection of mimics in the ex vivo vein organ model at 7 day time point (n=3).

Statistical analysis was performed using a paired t-test. P-values are included on the graphs: $^{**}P < 0.01$. n numbers correspond to distinct biological replicates.