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Local microRNA modulation using a novel anti-miR-21-eluting stent effectively prevents experimental in-stent restenosis

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Abstract

Objective—Despite advances in stent technology for vascular interventions, in-stent restenosis (ISR) due to myointimal hyperplasia (MH) remains a major complication.

Approach and Results—We investigated the regulatory role of microRNAs in MH/ISR, utilizing a humanized animal model in which balloon-injured human internal mammary arteries (IMAs) with or without stenting were transplanted into RNU rats, followed by microRNA profiling. miR-21 was the only significantly up-regulated candidate. In addition, miR-21

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expression was increased in human tissue samples from patients with ISR compared to coronary artery disease specimen. We systemically repressed miR-21 *via* intravenous FAM-tagged-LNA-anti-miR-21 (anti-21) in our humanized MH-model. As expected, suppression of vascular miR-21 correlated dose-dependently with reduced luminal obliteration. Further, anti-21 did not impede re-endothelialization. However, systemic anti-miR-21 had substantial off-target effects, lowering miR-21 expression in liver, heart, lung, and kidney with concomitant increased serum creatinine levels. We therefore assessed the feasibility of local miR-21 suppression using anti-21-coated stents. Compared to bare metal stents, anti-21-coated stents effectively reduced ISR, while no significant off-target effects could be observed.

Conclusion—This is the first study to demonstrate the efficacy of an anti-miR-coated stent for the reduction of ISR.

Keywords

Myointimal hyperplasia; restenosis; microRNA; local drug delivery; rat; humanized model

Introduction

A universal and common pathological process of cardiovascular diseases is myointimal hyperplasia (MH), characterized by augmented proliferation and migration of vascular smooth muscle cells (VSMC) and increased synthesis of extracellular matrix (ECM), which progressively obliterates the vessel lumen¹. These cellular events are the result of a dedifferentiation process of VSMCs, which are capable of losing their contractile function and re-establishing a more undifferentiated synthetic geno- and phenotype². This VSMC plasticity can be observed as a response to growth factors, cytokines, cell-cell contacts, lipids and ECM components and is defined by a distinct gene expression profile^{3, 4}. VSMCs further exhibit hyperpolarized mitochondria and an imbalanced replication-apoptosis ratio, which makes them highly proliferative⁵. Prevention of this VSMC phenotype holds promise for alleviating myointimal hyperplasia and subsequent obliterative vascular diseases like in-stent restenosis (ISR).

MicroRNAs (miRNAs) are small, endogenous antisense RNAs that regulate gene expression typically via mRNA degradation or translational repression⁶. miRNA manipulation can lead to broad alterations in regulatory pathways at multiple levels, as a single miRNA is capable of binding multiple mRNAs. These effects can be cell type- or tissue-specific. Previous studies suggest that miRNAs are critically involved in MH and ISR⁷. Modulation of various miRNAs can inhibit myointimal growth, suggesting a therapeutic approach for vascular disease^{7,8}.

Most *in vivo* studies examining MH have utilized rodent models, and systemic miRNA modulation carrying the risk for off-target effects⁹. In order to increase translational potential, we decided to utilize our previously described orthotopic transplant model^{5, 10}, and now report a novel strategy for local anti-miR (-21) delivery engaging coatable drug eluting stents (DES).

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

miR-21 is up-regulated during myointima formation

Balloon-injured human IMAs were implanted into the abdominal aortic position of athymic RNU rats (hMA model) to study MH as reported previously^{5, 10}. In a second cohort, each IMA had a single bare metal stent (BM-stent) deployed prior to implantation. Ten days post-implantation, we retrieved the IMAs from both groups (denoted hMA₁₀ and BM-stent₁₀, respectively) and measured tissue expression of eight miRNAs previously shown to be associated with the proliferative response in vascular (patho-)physiology (miR-1, -21, -29b, -133a, -143, -145, -221, and -222)^{8, 11}. Non-denuded native hMAs served as controls (“native”). Significant changes in miRNA expression were detected for all except one (miR-221) (Figure 1A and Figure I in the online-only Data Supplement). Except for miR-21 (significantly up-regulated), all other remaining miRNAs were substantially repressed. miRNA expression levels were similar in the hMA₁₀ and BM-stent₁₀ groups for all miRNAs measured. Importantly, miR-21 expression was low in native hMA, and non-stenosed human coronary arteries (hCA_{healthy}), while previously-stented, diseased human coronary arteries (hCA_{stent}) had similar miR-21 levels to the hMA₁₀/BM-stent₁₀ groups (Figure 1A), suggesting that our humanized *in vivo* model closely mimics human ISR. As miR-21 was substantially up-regulated, it appeared distinctly suitable for anti-miR-based therapeutic modulation.

SMA-positive myointimal cells in the hMA model co-localized with HLA I and did not express rat MHC I (Figure 1B), confirming human myointimal origin. SMemb (VSMC de-differentiation marker), and SM heavy chain (VSMC-specific marker) were found in the myointima of hMA₂₈ vessels (immunofluorescence; Figure 1C), as was miR-21 in comparison to un-denuded control hMA and non-stenosed human coronary arteries when analyzed by *in situ* hybridization (ISH; Figure 1D). miR-21 was approximately 4-fold up-regulated in injured human arteries, and abundantly expressed in the VSMC-rich human myointima.

Systemic miR-21 repression

We sought to inhibit miR-21 in the hMA model through systemic administration. RNU rats received one intravenous dose of either 1mg/kg anti-21 in the low-dose (LD) or 5mg/kg anti-21 in the high-dose (HD) group, one day after hMA implantation. Vessels were retrieved after 28 days for histologic evaluation (Figure 2A). Untreated hMA₂₈ arteries showed a circular, cell-rich myointima with increased extracellular matrix deposition. Myointimal area was reduced in both anti-21 treatment groups, an effect that was markedly more pronounced with HD treatment.

Confocal immunofluorescence staining revealed similar composition of the myointimas in all of the above groups (Figure 2B). Double immunofluorescence staining against SMA and Fibroblast activation protein (FAP) showed high myointimal SMC content with fibroblastic

cells indicating an area of active tissue remodeling. Quantification of luminal obliteration confirmed significantly more luminal preservation in the anti-21 HD group compared to both untreated controls and the anti-21 LD group (Figure 2C). Effective knockdown of miR-21 in the hMA vessels with anti-21 HD treatment was confirmed by qRT-PCR (Figure 2D).

We next evaluated miR-21 off-target knockdown in other organs. As expected, we observed significant reduction of miR-21 expression in kidneys (Figure 2E), as well as liver, heart, and lungs (Figure IIA in the online-only Data Supplement). While other measured serum markers did not change significantly, creatinine elevation coincided with kidney miR-21 repression, indicating that systemic anti-21 HD administration negatively affects kidney function in our specific RNU rat model (Figure 2F). As the LNA-antisense was FAM-tagged, we were able to demonstrate (by fluorescence imaging) heavy renal accumulation of systemically-delivered anti-21 (Figure 2G). This potentially toxic side effect of anti-21 in kidneys should be taken into account when considering translational systemic miR-21 inhibition.

Local miR-21 repression using anti-21-eluting stents

To minimize the above-noted off-target effects, we coated stents with anti-21 to permit local delivery to the injured hMA vessels. Successful stent coating with anti-21 was confirmed by FAM fluorescence and scanning electron microscopy (Figure III in the online-only Data Supplement). The total anti-21 amount coated onto each stent was similar to the systemic HD dosage (5mg/kg). Anti-21-stents were compared with otherwise identical BM-stents in the humanized model (Figure IIIB in the online-only Data Supplement), and ISR was assessed after 28 days. Trichrome staining revealed marked myointima formation within the BM-stent lumen, and much reduced myointima in anti-21-stented hMA (Figure 3A). Fixation and cutting protocols for stented vessels necessitated stent strut removal, leaving gaps adjacent to the internal elastic lamina. Despite this, ISR was clearly decreased in the anti-21-stent group as observed by histological quantification (Figure 3B). Optical coherence tomography (OCT) images obtained immediately after vessel retrieval and prior to fixation, also indicated markedly smaller myointimal growth within the anti-21-stent₂₈ lumen (Movie S1, Figure 3C). Effective anti-21 uptake in the stented vessels was confirmed by fluorescence-imaging and -intensity analysis (Figures 3D-E). Screening for off-target regulation did not reveal any miR-21 expression changes in kidney (Figure 3F), liver, heart, or lung (Figure IIB in the online-only Data Supplement). Further, serum creatinine was unaffected by anti-21-stent placement (Figure 3G) and fluorescence confirmed no accumulation of anti-21 in the kidneys (Figure 3H).

Anti-21 does not affect re-endothelialization

We investigated whether anti-21 treatment affects re-endothelialization of balloon-injured arteries. Lewis rats underwent balloon injury of their abdominal aortas and received the above-mentioned, highly effective anti-21 HD intravenously or remained untreated as control. Interestingly, after 28 days endothelial cell (EC)-specific RECA-1 staining revealed near complete re-endothelialization in both groups (Figures 4A-B). We also performed organ chamber experiments to assess the functional capacity of the regenerated endothelium

to regulate vasodilatation (Figure 4C). Both control and anti-21-treated aortas showed similar endothelial-dependent and -independent relaxation characteristics. Together, these results indicate that re-growth of functionally intact endothelium in denuded arteries occurs despite anti-21 treatment. Of importance, *in vitro* proliferation of human coronary artery ECs (hCAECs) remained unaffected by anti-21 after applying different stimuli, such as serum starvation or platelet derived growth factor (PDGF) treatment (Figures 4D-E). Interestingly, only forced overexpression of miR-21 (pre-21 treatment) substantially increased MTT assay-detected EC proliferation (in comparison to scr-miR and anti-21; Figures 4D-E), indicating a less prominent regulatory effect of miR-21 inhibition in vascular ECs, an effect that was previously reported by others as well^{7, 12}.

Anti-21 inhibits proliferation of smooth muscle cells in vivo, ex vivo and in vitro

To confirm the observed anti-proliferative effect of miR-21 repression in VSMCs *ex vivo*, we stimulated fresh pieces of IMA with PDGF, an important driving factor for MH¹³ and miR-21 up-regulation in SMCs¹⁴ and fibroblasts¹⁵. PDGF not only increased miR-21 expression (Figure 4F), but also decreased PTEN (Figure 4G), a well-established target gene of miR-21 in vascular remodeling⁷. Simultaneous anti-21 treatment diminished PDGF-mediated PTEN suppression, confirming the intermediary and crucial role of miR-21 (Figure 4G). Inhibition of miR-21 abolished the pro-proliferative effect of PDGF (and serum starvation) on human coronary artery SMCs, whereas miR-21 overexpression substantially induced the pro-proliferative response (Figures 4H-I).

Conventional immunohistochemical analysis of explanted IMAs from our humanized *in vivo* model, in which rats were treated with systemic (high and low dose) anti-21, confirmed the inhibitory effect of the anti-21 treatment on SMC proliferation *via* de-repression of PTEN (Figure IV in the online-only Data Supplement). Transmural inflammation (as indicated by CD68-positive cells) was not impeded by anti-21 treatment in our model (Figure IV in the online-only Data Supplement).

Discussion

The treatment of patients with ISR remains challenging, despite recent advances in device technology and pharmacological treatment. BM stents are still widely used during percutaneous coronary interventions (PCI), despite their considerably high risk of ISR, especially when utilized for complex pathologic lesions or difficult anatomic scenarios¹⁶. The use of anti-proliferative drug-eluting stents (DES) substantially decreased myointimal proliferation and thus ISR. Several generations of DES have entered the clinic and markedly reduced the burden, particularly of early ISR. However, late ISR after DES placement remains a significant problem associated with delayed re-endothelialization due to cell-unspecific effects and prolonged thrombogenicity¹⁷. Thus, the discovery of highly effective treatments that allow local delivery and mainly target SMC-dominant MH, while not interrupting the re-endothelialization process, remain very appealing. The major aim of this study was to technically assess the feasibility of using miR modulation locally delivered from a DES to prevent a certain vascular response to injury, in our case ISR.

Vascular remodeling is a term widely used for adaptive structural changes of the vasculature to different types of injury. Myointima formation may develop after PCI and stenting of diseased arteries and leads to ISR. Numerous miRNAs have previously been suggested to play a role in proliferation-associated vascular diseases. Out of 8 miRNA candidates assessed in this study, all except for one miRNA (miR-221) were substantially altered in injured (hMA groups) as well as stented hMAs (BM-stent groups) compared to controls. All miRNAs but miR-21 (increased expression) were significantly reduced in response to injury.

The other miRNAs that were down-regulated included the muscle cell-enriched miR-1 and miR-133, which are crucial mediators in muscular development and in response to vascular injury¹⁸. miR-29b, which has been identified as an important mediator of the fibrotic response in the vasculature by directly targeting several collagen isoforms as well as other important components of the extracellular matrix such as elastin and fibrillin1^{19, 20}. The miR-143/-145 cluster is particularly important in its ability to perform phenotypic switching in VSMCs in response to vascular injury^{21, 22}. MiR-221 and miR-222 have been shown to be prominently involved in vascular proliferation by targeting p27 (Kip1) and p57 (Kip2) as well as the stem cell factor c-Kit in VSMCs²³.

Of importance for treatment considerations, miR-21 was the only miRNA in our study that was significantly up-regulated in response to injury. miRNAs with augmented expression in response to injury and disease are amenable for antisense oligonucleotide (anti-miR) inhibition and may therefore present promising therapeutic targets on the molecular level. Therapeutic overexpression of suppressed miRs is currently less attractive, and the delivery of miRNA-mimics or pre-miRNAs for miRNA induction remains problematic. While LNA-based miRNA-mimics are becoming available in the near future, traditional delivery has involved lenti- or adenoviral approaches, presenting risks common to gene therapy²⁴. Some newer studies from the cancer field have explored packaging synthetic miRNA duplexes within lipid nanoparticles, and have been quite successful with this approach in murine models²⁵.

A major difference between the classical pharmacologic approach, and the novel field of miRNA-based therapeutics is that the latter may regulate entire gene or protein networks⁸. Recent animal and human efficacy data are promising, suggesting that anti-miRs have the potential to become a new class of drugs^{26,27}. Anti-miRs have several advantages for drug development, including their small molecular size and the frequent conservation of their target miRNAs across species⁶.

miR-21 is considered to be among the most readily inducible vascular miRNAs, associated with increased cell proliferation and decreased apoptosis in the vessel wall^{28,12}. In a previous study, antisense-mediated inhibition of miR-21 using pluronic gel reduced myointima in balloon-injured rat carotid arteries⁷. The authors showed that PTEN and Bcl-2 up-regulation were involved in the anti-miR-21-mediated cellular effects⁷. Similarly, in vein graft failure studies, miR-21 was significantly up-regulated following engraftment in mouse, pig, and human models. Genetic deletion of miR-21 in mice and grafted veins limited the proliferative response and reduced myointima formation²⁹.

Our anti-21-eluting stent was highly effective in reducing VSMC proliferation and alleviating myointimal hyperplasia and ISR. Importantly, we did not observe miR-21 suppression in other organs, nor did we observe off-target side effects with this approach. Anti-21, in contrast to the currently employed DES medications such as rapamycin³⁰, did not delay vessel re-endothelization, a side effect believed to be the major cause of stent thrombosis and late ISR¹⁶. In our study, systemic anti-21 allowed re-coverage of balloon-injured rat aortas with functionally intact endothelium. In addition, anti-21 treatment did not inhibit EC proliferation *in vitro*. This anti-21 effect is most likely due to the very low concentration of miR-21 in the endothelium compared to its expression levels in VSMC, being particularly enriched in the myointima and vessel media in response to injury and stent deployment¹².

Off-target effects in organs in which miRNA-modulators assimilate to a much higher extent than the targeted vasculature are a much-debated compromise of antisense oligonucleotide therapeutics. Anti-miR-21 treatment in our ISR studies increased creatinine levels in RNU rats, which might be due to the immune-incompetent state of the rats, and thus model-specific. Of note, previous studies evaluating the development of kidney fibrosis in mice showed protective effects of miR-21 inhibition^{31, 32}. Of importance, these studies were mainly conducted in diseased mice to prevent or reverse kidney fibrosis, and thus were not performed in un-diseased and otherwise healthy rodents. miR-21 plays an important role in controlling cell apoptosis in various cells types (such as tubular epithelial cells; TEC). Inhibition of miR-21 in TEC increases cell death³³⁻³⁵, which could be an explanation of our observation in the RNU rats.

We believe this proof-of-concept and technical assessment study for the efficacy and safety of anti-21-eluting stents demonstrates the feasibility of local delivery for miRNA-based therapeutics being applicable for vascular diseases. The method could potentially be optimized by modulating the release kinetics and dosing of the anti-miR, or by potentially combining multiple anti-miRs on a single stent. Future trials should address these issues, as well as its long-term efficacy in preventing the occurrence of late ISR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMS	bare metal stent
CAD	coronary artery disease
DES	drug eluting stent
ECM	extracellular matrix
FAM	fluorescein
FAP	fibroblast activation protein alpha
hCA	human coronary artery
HD	high dose
HLA I	human leukocyte antigen I
hMA	humanized mammary artery model
IMA	internal mammary artery
ISH	in situ hybridization
ISR	in-stent-restenosis
LD	low dose
LNA	locked nucleic acid
MH	myointimal hyperplasia
MHC I	major histocompatibility complex I
miR	microRNA
mRNA	messenger RNA
OCT	optical coherence tomography
PDGF	platelet derived growth factor
qRT-PCR	quantitative real time polymerase chain reaction
RNU	Rowett nude (rats)
SMA	smooth muscle cell α -actin
VSMC	vascular smooth muscle cell

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Significance

microRNA-based therapeutics have the potential of becoming a whole new class of drugs. However, the systemic form of their administration carries a substantial risk for off-target effects on organ systems (e.g., liver, kidney), in which these modulators assimilate to a much higher extent than the targeted tissue, such as the vasculature in our case. The utilization of local forms of delivery, such as drug eluting stents could significantly elevate the therapeutic potential of microRNA modulation in cardiovascular pathologies.

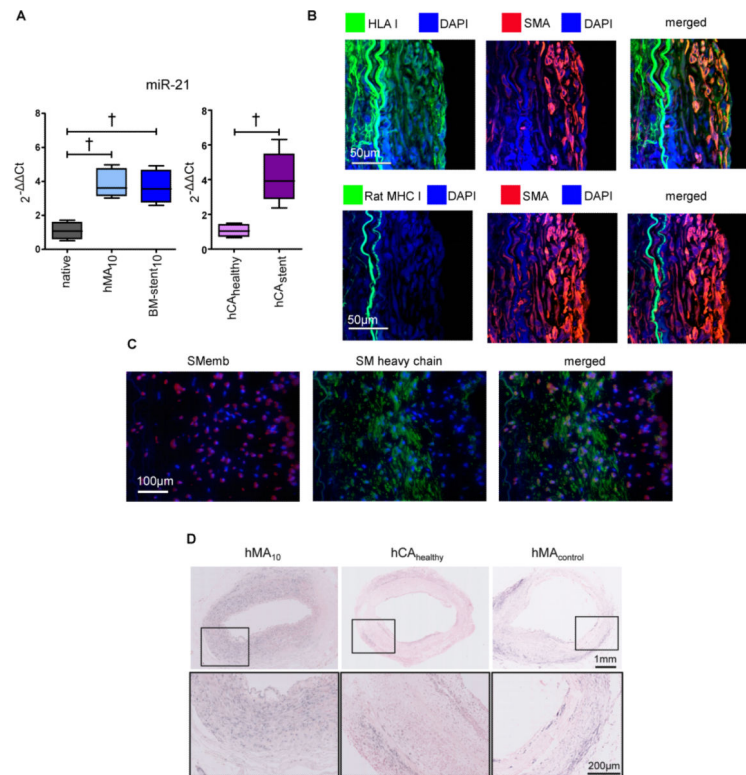


Figure 1. MiR-21 is involved in human myointimal hyperplasia

(A) Human IMAs underwent either balloon denudation (hMA), or denudation and stenting (BM-stent) and were implanted into the abdominal aortic position of RNU rats to await myointima development. After 10 days, qRT-PCR measurement of miR-21 revealed significant overexpression in both hMA and BM-stent groups as compared to native IMA (n=10 (native), n=6 (hMA), n=6 (BM-stent), ANOVA with Bonferroni's post-hoc test). Likewise, miR-21 levels in diseased, stented human coronary artery (hCA_{stent} n=5;) were similarly altered vs. normal vessels (hCA_{healthy} n=4; unpaired t-test). (B) Human origin of myointimal cells in the hMA model was confirmed by double-positive immunofluorescence staining for SMA and human leukocyte antigen class I (HLA I) and the lack of rat major histocompatibility complex class I (rat MHC I). (C) Smooth muscle cells (SMC) within the myointima of hMA₂₈ showed expression of SMemb and/or SM heavy chain. Synthetic, SMemb positive cells were distributed more in neo-myointimal regions and SM-heavy chain positive, contractile SMCs were found near the elastic laminae. (D) Presence of miR-21 in hMA₁₀ confirmed by *in situ hybridization* in comparison to un-denuded hMA (hMA_{control}) and uninjured healthy human coronary arteries (hCA_{healthy}; purple chromagen). † p<0.01.

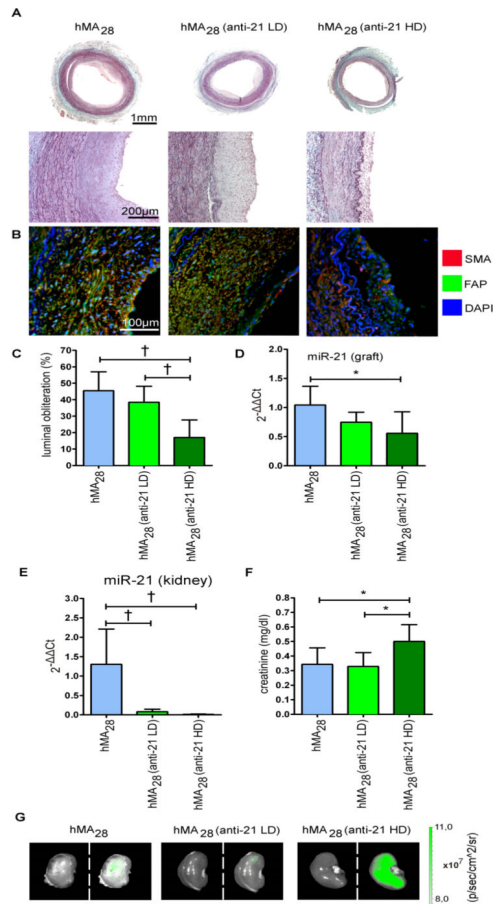


Figure 2. Systemic modulation of miR-21 inhibits myointima development, but causes off-target effects

(A) To assess the effect of miR-21 modulation on myointimal hyperplasia, 1mg/kg (LD), 5mg/kg (HD) anti-miR-21 (anti-21) or vehicle were injected intravenously one day post-IMA-implantation. After 28 days, hMA-vessels were excised and stained with Masson's Trichrome. (B) Double immunofluorescence staining against SMA and FAP demonstrates similar myointimal composition in all groups. (C) Analysis of hMA vessels revealed marked luminal obliteration in the non-treated group, while anti-21-treated vessels showed less obliteration in a dose dependent manner (mean \pm s.d., n=7 animals per group, ANOVA with Bonferroni's post-hoc test). (D) Sufficient inhibition of miR-21 in hMA vessel through anti-21 was confirmed by qRT-PCR. (mean \pm s.d., n=7 (hMA), n=6 (anti-21 LD), n=6 (anti-21 HD), ANOVA with Bonferroni's post-hoc test). (E) Systemic miR-21 inhibition caused marked reduction of miR-21 expression in kidneys (mean \pm s.d., n=6 (hMA), n=7 (anti-21 LD), n=7 (anti-21 HD), ANOVA with Bonferroni's post-hoc test). (F) This correlated with an increase in creatinine (mean \pm s.d., n=7 animals per group, ANOVA with Bonferroni's post-hoc test). (G) Injected anti-21 was tracked via fluorescence imaging. High fluorescence signal was detected in HD-treated rat kidneys, indicating an accumulation of anti-21 (left: photo picture; right: overlay picture with fluorescence signal). * p<0.05, † p<0.01.

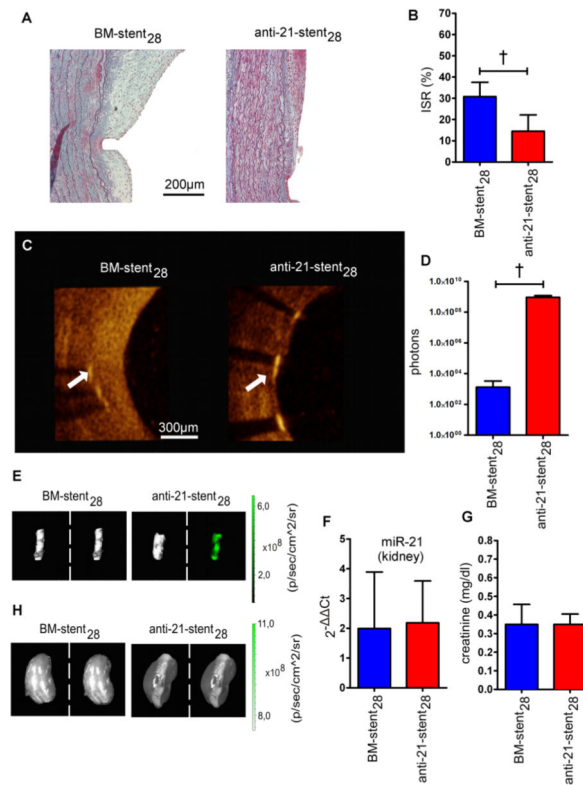


Figure 3. Local inhibition of miR-21 prevents myointima development without exerting off-target effects

(A) For local miR-21 inhibition, anti-21-coated stents were expanded into denuded IMAs, which were then implanted into RNU rats. Histological sections of stented vessel stained with Masson's Trichrome are shown 28 days after implantation. (B) Anti-21-stent coating resulted in significantly less luminal obliteration compared to the BM-stent group (mean± s.d., n=8 (BM-stent), n=6 (anti-21-stent), unpaired t-test). (C) OCT images revealed considerably less myointima formation in the anti-21-stent group (white arrow marks a stent strut, luminal side is to the right). (D) Fluorescent signal intensity measurements confirm anti-21 uptake in the anti-21-stent group (mean± s.d., n=5 per group, unpaired t-test). (E) Corresponding representative fluorescence images to signal intensity measurements shown in D. (F) miR-21 levels in kidneys were not affected by anti-21-coated stent delivery (mean± s.d., n=6 per group, unpaired t-test) and (G) serum creatinine showed no significant difference between the BM-stent and anti-21-stent groups (mean± s.d., n=8 (BM-stent), n=6 (anti-21-stent), unpaired t-test). (H) In fluorescence imaging, similar signals were detected in kidneys of both groups (left: photo picture; right: overlay picture with fluorescence signal). † p<0.01.

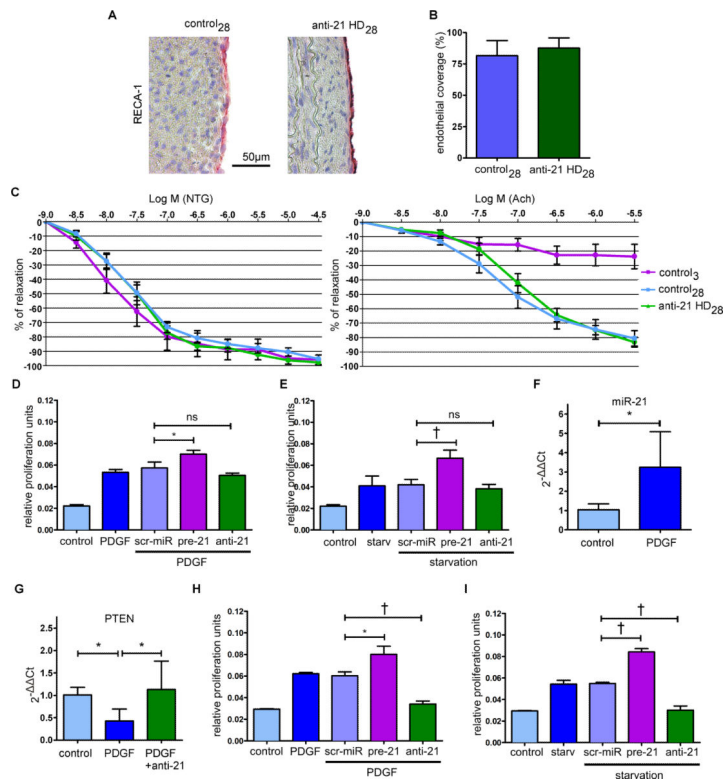


Figure 4. Anti-21 treatment does not impair re-endothelialization and abolishes stimulatory proliferative effects on SMCs in vitro

(A) To assess the effect of anti-21 on vessel re-endothelialization, rat aortas underwent endothelial denudation via balloon injury. Animals in the treatment group received 5mg/kg anti-21 intravenously one day post-denudation. After 28 days, vessels were recovered and stained for endothelial cells. (B) There were no relevant differences in endothelial coverage between control₂₈ and anti-21₂₈ (mean± s.d., n=5 per group, unpaired t-test). (C) Endothelial function was assessed in organ chamber experiments (mean± SEM, n=6 (control₂₈), n=6 (anti-21 HD₂₈), n=3 (control₃)). All three groups showed similar endothelial-independent relaxation characteristics, when stimulated with nitroglycerine. In contrast, stimulation with acetylcholine had no effect 3 days after vessel injury, while both control₂₈ and anti-21 HD₂₈ groups showed similar physiologic endothelial-dependent relaxation. (D) Proliferation assays with hCAECs after PDGF or 48h serum starvation (E) treatment revealed a negligible effect of miR-21 inhibition on EC proliferation (mean± s.d., quadruplicates of 3 independent experiments, unpaired t-test). (F) To determine the effect of PDGF on miR-21 expression in vessels, fresh pieces of IMA were stimulated with PDGF. 24h post-stimulation, miR-21 expression was significantly elevated (mean± s.d., n=6 (control), n=10 (PDGF), unpaired t-test) (G), while PTEN expression was suppressed. Concurrent transfection with anti-21 abolished this effect (mean± s.d., n=5 (control), n=5 (PDGF), n=4 (PDGF+anti-21), ANOVA with LSD post-hoc test). (H) Inhibition of miR-21 with anti-21 caused diminished proliferation in PDGF-stimulated hCASCs and (I) after 48h of serum starvation (mean± s.d., quadruplicates of 3 independent experiments, unpaired t-test). * p<0.05, † p<0.01.