Materials and Methods (Supplement Only)

Human sample acquisition

Remnant segments of human internal mammary arteries (IMAs) from routine coronary artery bypass surgeries, and stented coronary vessels retrieved from explanted native hearts of heart transplant recipients were obtained. Their use for research was approved by the Institutional Review Board (IRB), and patients gave written informed consent.

Rats

Male athymic RNU-rats (Crl:NIH-Foxn1^{rnu}) and Lewis rats were purchased from Charles River (Sulzfeld, Germany). Animals were housed in the animal care facilities of the University Hospital Hamburg-Eppendorf and received humane care in compliance with the Guide for the Principles of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, and published by the National Institutes of Health. All animal protocols were approved by the local authority (''Amt für Gesundheit und Verbraucherschutz, Hansestadt Hamburg'').

Humanized mammary artery denudation (hMA) and stent implantation model

IMA endothelium was removed by balloon injury using a 2-French (Fr) Fogarty catheter (Suppl. Fig. 3B) as described before[1, 2]. Briefly, a segment of the abdominal aorta of RNU-rats is removed and replaced by the denuded human IMA via end-to-end anastomosis using 8-0 sutures (Ethicon, Norderstedt, Germany). In this model we do not detect any signs of advanced cell death and/or proliferation, as well as altered inflammatory activation in the IMA-recipient RNU rats. Stent implantation was performed as indicated in Suppl. Fig. 3B, using either bare metal stents (BM-stent) or anti-miR-21-eluting stents (anti-21-stent). 2.75mm x 8mm stents (Translumina, Hechingen, Germany) were deployed into balloon-injured IMAs, which were then implanted into the abdominal position of RNU rats as previously described by our group[1, 2]. Implanted vessels were harvested on the day indicated in the subscript.

Rat aortic denudation model

To study vessel re-endothelialization after balloon injury with and without systemic anti-21 treatment, endothelial denudation in male Lewis rats was performed via balloon inflation as previously described[1]. Vessels from all models described above were retrieved at the postoperative day indicated in the subscript number after the group name.

Systemic anti-21 treatment in vivo

FAM-tagged locked nucleic acid (LNA) anti-miR-21 (anti-21) (Exiqon, Vedbaek, Denmark; 5'-3': 56-FAM-/TCAGTCTGATAAGCT) was dissolved in PBS. Animals were treated with one dose of anti-21 via intravenous femoral vein injection, one day after vessel implantation. The concentration of anti-21 was 1mg/kg in the low-dose (LD) group and 5mg/kg in the high-dose (HD) group.

Anti-21 stent coating

The Translumina YUKON DES System with PEARL microporous surface was used as previously described[3]. Animals were weighted prior to stent coating and 5mg/kg animal weight of FAM-tagged-LNA-anti-miR-21 (Exiqon) were dissolved in 70% isopropyl alcohol and coated onto the stent. The coated stent surface was visualized with a fluorescence

microscope (Leica, Wetzlar, Germany) and a Hitachi S-4800 scanning electron microscope (Hitachi, Düsseldorf, Germany).

Histological analysis

All arteries were perfusion fixed with 4% paraformaldehyde to ensure full distension before performing quantification if applicable. Tissues were then dehydrated, and embedded in paraffin. Stented grafts were fixed and dehydrated alike and infiltrated in a mixture (MMA I) of 59.3% methyl methacrylate, 34.6% butyl methacrylate, 4.9% methyl benzoate and 1.2% polyethylene glycol for 2 days, MMA I with 0.4% dry benzoyl peroxide for 2 days, and MMA I with 0.8% dry benzoyl peroxide (MMA III) for 2 days at 4°C. Subsequently, grafts were embedded on a pre-polymerized base in a solution of MMA III with N,N-dimethyl-p-toluidine. Continuous polymerization was conducted in a climate chamber (Binder, Tuttlingen, Germany). Slides of 5µm thickness were cut and stained with Masson's trichrome for morphometric analysis of myointimal lesion. Luminal obliteration was defined as the percentage of the cross-sectional area within the internal elastic lamina taken up by the intima.

Immunofluorescence staining

Rehydrated tissue slides underwent heat-induced antigen retrieval with Dako antigen retrieval solution (Dako, Glostrup, Denmark) in a steamer, followed by antigen blocking with Image-iT® FX signal enhancer (Invitrogen, Carlsbad, CA). Antibodies against smooth-muscle-actin (SMA, ab5694; Abcam, Cambridge, UK), smooth-muscle-myosin-heavy-chain (EPR5335; Abcam), SMemb (3H2; Yamasa, Tokyo, Japan), fibroblast-activation-protein (FAP, ab28246; Abcam), human leukocyte antigen I (HLA I, 3F10; Santa Cruz, Santa Cruz, CA), and rat FITC-conjugated major histocompatibility complex I (MHC I, B5; BD Pharmingen, Franklin Lakes, NJ) were incubated for 1h at 37°C. After washing with PBS, sections were incubated for 1h at 37°C with a corresponding secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen). Cell nuclei were counterstained with DAPI and imaging was performed using a Nikon Eclipse TiE microscope (Nikon, Tokyo, Japan) equipped with the Perkin Elmer UltraVIEW VoX confocal imaging system (Perkin Elmer, Waltham, MA)

Fluorescence imaging

To demonstrate overall anti-21 distribution, freshly recovered organs (heart, lung, liver, kidney, stented or un-stented vessels) were washed in PBS and imaged using a Xenogen IVIS200 imaging system (Caliper Lifesciences, Hopkinton, MA). Fluorescent signal of FAM-tagged anti-21 was visualized at 488nm in relative light units (photons/s/cm^2).

In situ hybridization

The miRCURY LNA microRNA ISH Optimization Kit (Exiqon) was used for *in situ* hybridization (ISH) of miR-21. Staining was performed according to manufacturer's instructions and as previously described[4]. In brief, tissue sections were treated with Proteinase K followed by a two-hour hybridization at the appropriate temperature. After washing in saline-sodium citrate buffers, we applied standard DIG blocking and staining procedures. Nuclear counterstaining was done with Nuclear Fast Red (Sigma Aldrich, St. Louis, MO).

Immunohistochemistry
Arterial tissue was stained with rabbit antibodies against PTEN, SMA, CD68, and KI67 (all from Abcam) using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for immunohistochemical analysis. The endothelium was stained using antibodies against RECA-1 (HIS52; Serotec, Raleigh, NC) and the ZytoChem-Plus AP Polymer-Kit (Zytomed Systems, Berlin, Germany). Histological analyses were obtained at room temperature using a Zeiss Axioplan 2 (Carl Zeiss MicroImaging, Jena, Germany) with Zeiss Achroplan and Zeiss Plan-Neofluar lenses, a Nikon Digital Sight (DS) Ri1 camera and the NIS-Elements F 3.00 software (Nikon, Tokyo, Japan).

Off-target effect screening
Rat blood was collected during vessel retrieval, and the following parameters were measured at University Hospital Hamburg-Eppendorf using routine techniques: aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), and triglycerides. At sacrifice, rat hearts, lungs, livers, and kidneys were harvested for fluorescent microscopy, RNA extraction, and qRT-PCR.

Organ chamber and ex vivo IMA ring assays
Freshly harvested aortae were cut into rings of 4mm size, and endothelium-dependent and -independent relaxation was measured as previously described[1]. Fresh pieces of IMA were denuded using 2-Fr Fogarty catheter, cut into rings of 2mm size, and kept in RPMI medium 1640 (Gibco, Carlsbad, CA) containing 10% fetal bovine serum. PDGF-BB (Sigma) at 200 ng/ml and/or anti-21 at 10µg/ml were added to the IMA-pieces and incubated for 24h at 4°C.

Optical coherence tomography (OCT) imaging
OCT images were recorded using the M2 OCT imaging system (LightLab Imaging, Westford, MA) as described previously[5].

RNA extraction and quantitative real-time PCR
Total RNA was isolated using the miReasy mini kit (Qiagen, Hilden, Germany). Tissues were disrupted using a tissue homogenizer (ProScientific, Oxford, CT), or stainless steel beads (5mm) in combination with TissueLyser (Qiagen). RNA quantity and quality was assessed using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE; 260/280 ratio 1.9-2.1). RNA was reverse-transcribed using the TaqMan microRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. MicroRNA Assay Kits (Life Technologies) for hsa-miR-1 (UGGAAUGUAAAGAAGUAUGU AU), hsa-miR-21 (UAGCUUAUCAGACUGAUGUUGA), hsa-miR-29b (UAGCACCAUUUGAAAUCAGUGUU), hsa-miR-133a (UUUGGUCCCCU UCAACCAGCUG), hsa-miR-143 (UGAGAUGAAGCACUGUAGCUC), hsa-miR-145 (GUCCAGUUUUCCCAGGAAUCCCU), hsa-miR-221 AGCUACAUUG UCUGCUGGGUUUC, hsa-miR-222 (AGCUACAUCUGGCUACUGGGU) and U6, RNU44, RNU48 (endogenous controls for normalization in human) and U6 and U87 (endogenous controls for normalization in rat samples) were used. For mRNA, the High Capacity RT kit with Rnase out (Life Technologies) was used to synthetize first-strand cDNA according to the manufacturer's protocol. qRT-PCR was performed using rat and human specific primers for PTEN (Life Technologies). Probes were normalized to RPLP0 (human) or HPRT1 (rat) as internal controls. Amplification took place on an ABI PRISM 7900HT (Applied Biosystems, Carlsbad,

CA). All data were calculated using the method of ΔΔCt values and are expressed as mean ± SD.

Proliferation assay

The MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Vybrant, Life Technologies) was performed following the manufacturer's instructions. Anti-21 treatment group was compared to the scrambled miR control group. After cell transfection, cells were incubated for 4 h in the presence of 10µl of MTT AB solution (Millipore, Billerica, MA). The formazan product was dissolved through adding 100µl acidic isopropanol (0.04N HCl) and absorbance was measured at 570 nm (reference wavelength 630 nm) on an ELISA plate reader.

In vitro studies

Human coronary artery smooth muscle cells (hCASMCs) and human coronary artery endothelial cells (hCAECs) were propagated in appropriate growth media in standard culture conditions with 5% FBS (Lonza, Basel, Switzerland; passage 5 to 6). Cells were either serum-starved or treated with PDGF for 48 h prior to transfection. Cells were transfected at 70-80% confluency with anti-21, or mismatch (scrambled) miRs using Lipofectamine RNAiMAX (all from Life Technologies). The concentration of the pre-diluted 50µM anti-miR-21 solution to transfect cells (for 12h) was 150pmol/plate (6-well plates) as recommended by the manufacturer's protocol, and as previously described by us[4]. Experiments were carried out in triplicates.

Statistical analysis

Data are presented as mean ± standard deviation or mean ± standard error of the mean as indicated. Analysis was carried out using SPSS statistical software package 22.0 (SPSS, Chicago, IL). Groups were compared with the unpaired Student's t-test, or analysis of variance (ANOVA) with Bonferroni's or LSD post-hoc test as indicated. Significance is displayed as * $p<0.05$ and † $p<0.01$.

References

1. Deuse T, Hua X, Wang D, Maegdefessel L, Heeren J, Scheja L, et al. Dichloroacetate prevents restenosis in preclinical animal models of vessel injury. *Nature*. 2014;509:641-644
2. Hua X, Deuse T, Michelakis ED, Haromy A, Tsao PS, Maegdefessel L, et al. Human internal mammary artery (ima) transplantation and stenting: A human model to study the development of in-stent restenosis. *Journal of visualized experiments : JoVE*. 2012:e3663
3. Deuse T, Erben RG, Ikeno F, Behnisch B, Boeger R, Connolly AJ, et al. Introducing the first polymer-free leflunomide eluting stent. *Atherosclerosis*. 2008;200:126-134
4. Maegdefessel L, Azuma J, Toh R, Deng A, Merk DR, Raiesdana A, et al. Microrna-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion. *Science translational medicine*. 2012;4:122ra122
5. Deuse T, Koyanagi T, Erben RG, Hua X, Velden J, Ikeno F, et al. Sustained inhibition of epsilon protein kinase c inhibits vascular restenosis after balloon injury and stenting. *Circulation*. 2010;122:S170-178