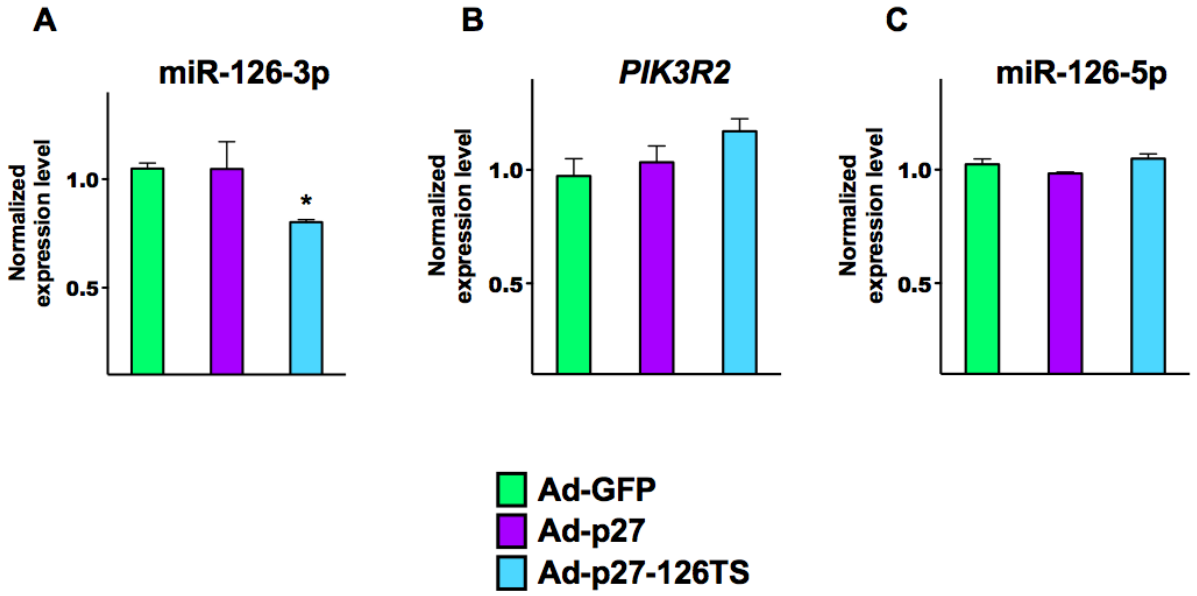
ONLINE DATA SUPPLEMENTS

Santulli G. et al. **"A microRNA-based strategy to suppress restenosis while preserving endothelial function"**

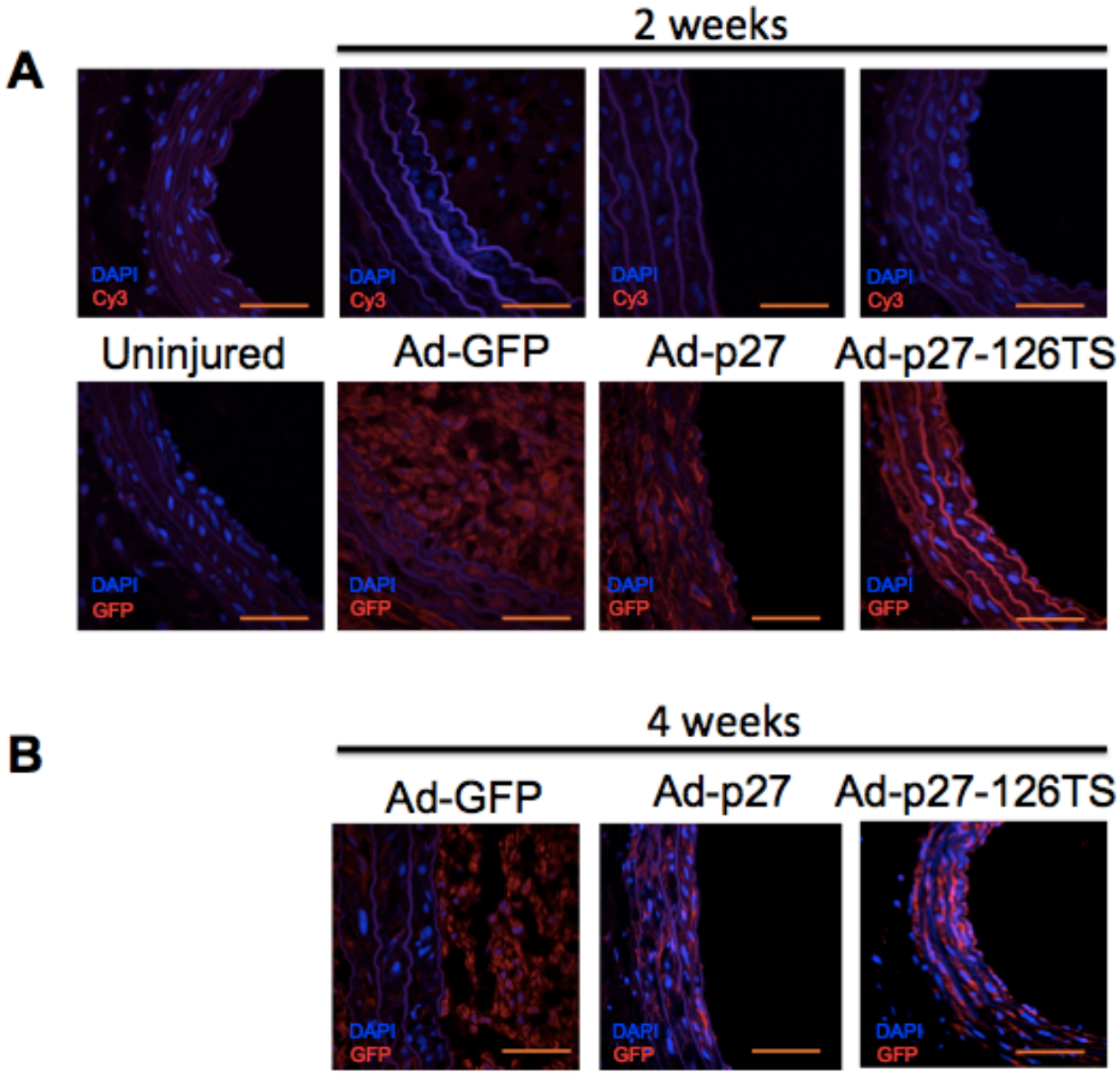
Supplementary Figures

Figure S1



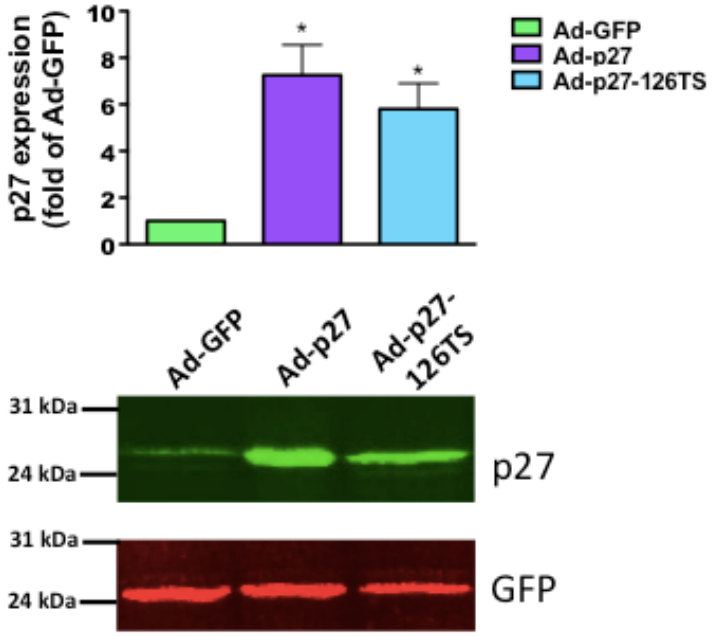
Effect of Ad-p27-126TS on the expression of miR-126-3p, *PIK3R2* and miR-126-5p in vitro.
EC were infected with the indicated adenoviruses for 24 h. Total RNA was extracted, and real time RT-qPCR for 126-3p **(A)**, *PIK3R2* **(B)**, and miR-126-5p **(C)** was performed. Expression levels of miRNAs were normalized to U18, *PIK3R2* was normalized to *GAPDH*. Data shown are means±SE; *p<0.05 vs Ad-GFP, one-way ANOVA, Tukey-Kramer *post hoc* test.

Figure S2



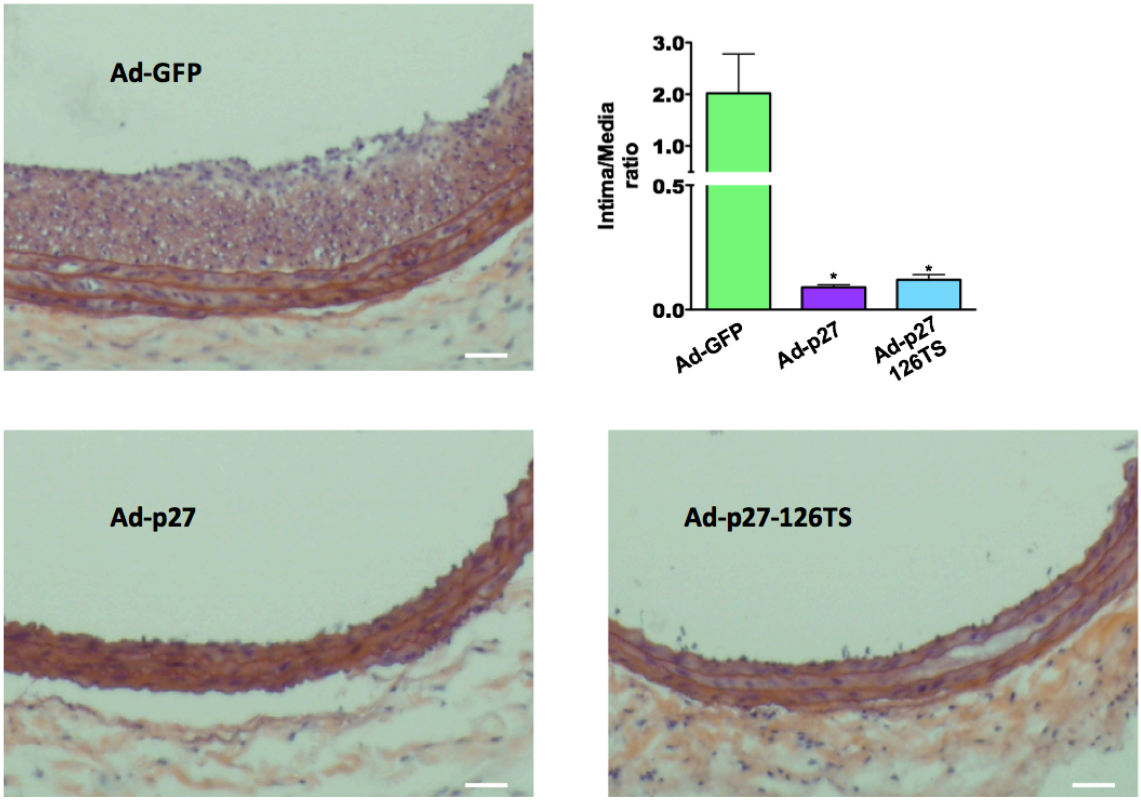
Efficiency of Ad infection two and four weeks after surgery.
The efficiency of Ad infection was evaluated 2 (**A**) and 4 weeks after surgery (**B**) using a primary antibody against GFP detected using a Cy3-coniugated secondary antibody. Representative digital images are shown. In A, top: negative control (secondary antibody alone, Cy3); bottom: staining for GFP revealed by Cy3. Scale bar represents 100 μm (magnification 60×).

Figure S3



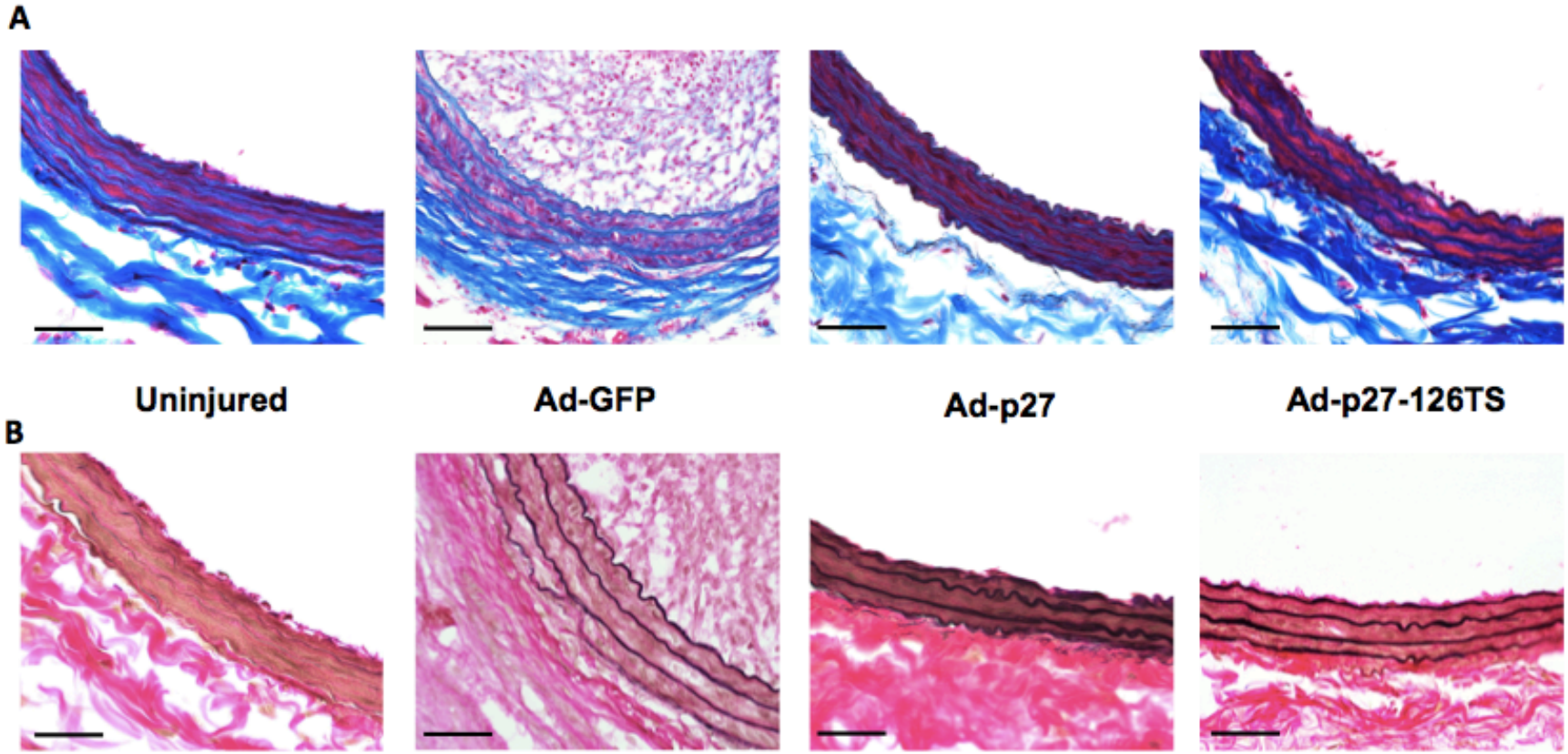
Assessment of p27 overexpression in the carotid wall three days after surgery. Three days after infection with the indicated Ad constructs the vessels were homogenized and samples were size-fractionated by SDS-PAGE; proteins were transferred to polyvinylidene difluoride membrane and visualized by immunoblotting using infrared-labeled antibodies. Data shown in the graph represents means±SE; *p<0.05 vs Ad-GFP, one-way ANOVA, Tukey-Kramer *post hoc* test.

Figure S4



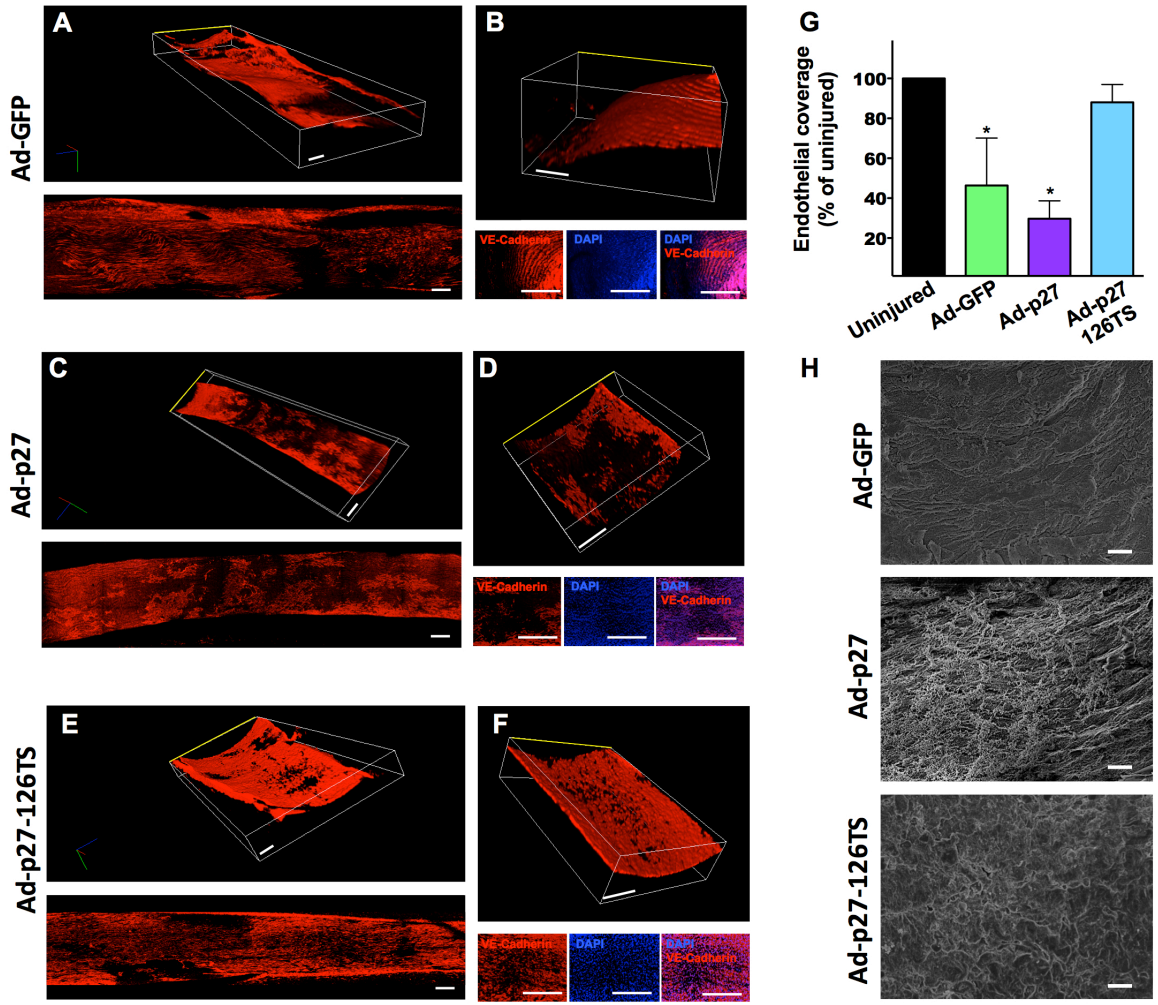
Ad-p27 and Ad-p27-126TS inhibit restenosis 4 weeks after balloon injury. Representative sections stained with hematoxylin / eosin (magnification 40×; dimensional bar represents 100 μm). Intima/media ratios were calculated from at least 6 rats/group. All data shown are means ± SEM. Data comparisons were made using one way ANOVA followed by Tukey-Kramer *post hoc* test; **P*<0.01 versus Ad-GFP.

Figure S5



Ad-p27 and Ad-p27-126TS restored the arterial structure to the uninjured control vessel.
Representative sections stained with **(A)** Masson's Trichrome to detect collagen fibers or **(B)** Verhoeff Van Gieson to detect elastin fibers (magnification 40×; dimensional bar represents 100 μm).

Figure S6.

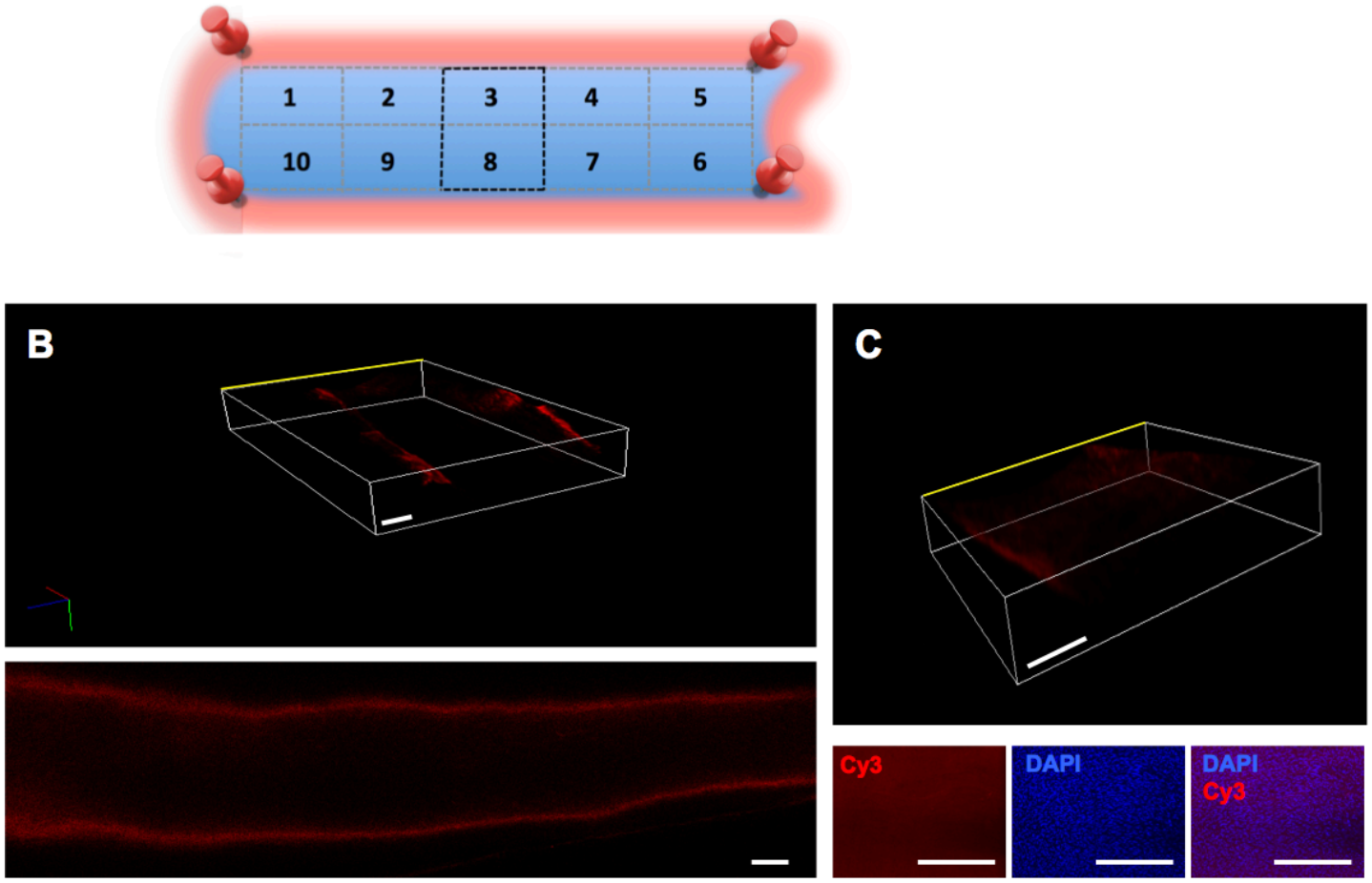


Endothelial coverage assessed by confocal electron microscopy of *en face* longitudinal arterial preparations 4 weeks after injury.

(**A-F**) Representative confocal images of the internal surface of the vessels immunostained for VE-Cadherin acquired at the. (**A, C, E**) Tridimensional (top) and bidimensional (bottom) imaging of the longitudinal *en face* preparation of the carotid arteries. (**B, D, F**) Tridimensional imaging of a representative central portion (see the methods and **Supplementary Fig. 7A** for detailed information about the imaging procedure) of the vessel (top), with the respective 2D

pictures showing the EC-specific immunostaining for VE-cadherin (bottom: VE-cadherin, DAPI and merge, as indicated), representing the endothelial coverage, which is quantified in panel **G**; the luminal side is indicated by the yellow line; see also the **Supplementary videos 9-14** for a tridimensional view. In all 2D and 3D pictures the dimensional bar is 400 μm. All data shown are means ± s.e.m. Data comparisons were made using one-way ANOVA with Tukey-Kramer *post hoc* test; n = at least 5/group; **P*<0.01 versus uninjured. (**H**) Representative scanning electron microscopy (SEM) images of the internal surface of uninjured, Ad-GFP, Ad-p27 and Ad-p27-126TS infected arteries. In SEM pictures, the scale bar indicates 10 μm.

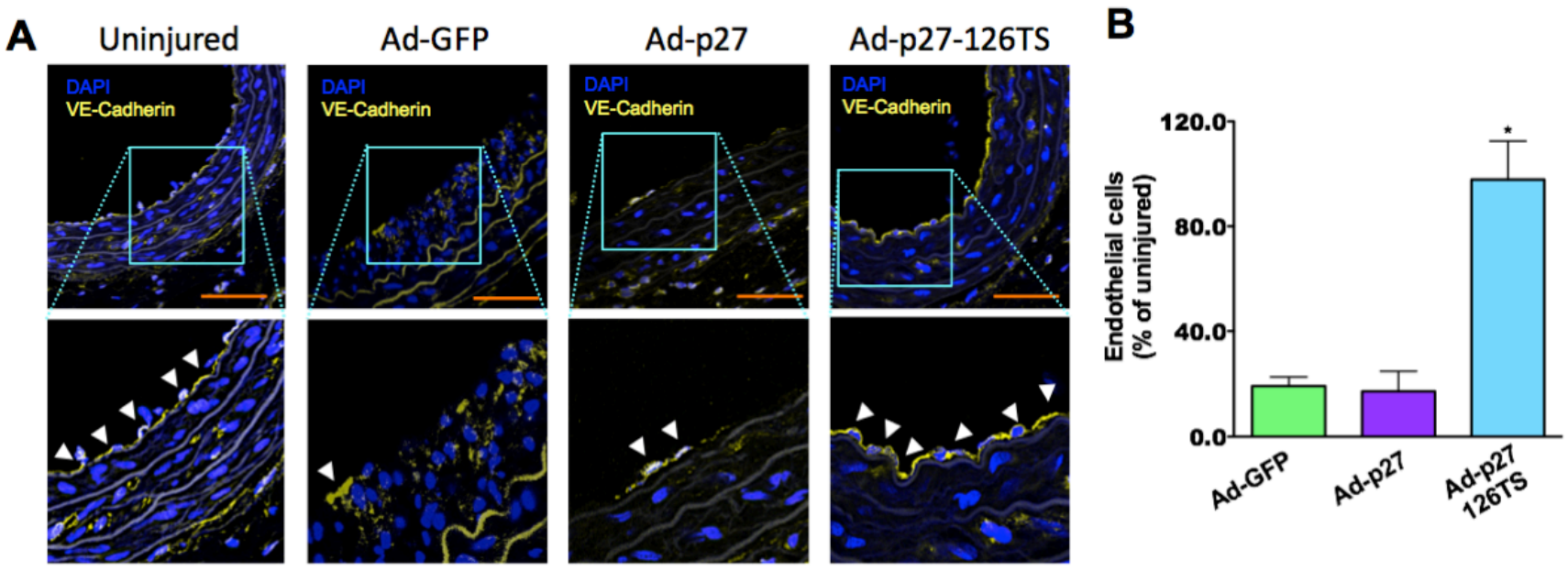
Figure S7



Imaging and quantifications of the longitudinal *en face* arterial preparations.
(**A**) Schematic representation of the examination of en face preparations at the confocal microscope. Ten adjacent quadrants of each carotid artery were independently scanned and subsequently 3D reconstructed using a dedicated software (Nikon NIS-Elements) to obtain the image of the whole vessel. The quantification of the endothelial coverage (VE-Cadherin staining) was performed quantifying the red areas of the central portion of the artery (represented by bold dashed lines, quadrants 3 and 8) by using ImageJ64. (**B**) No specific staining is shown (20× magnification, confocal microscope) in the whole uninjured vessel stained with the Cy3 secondary antibody alone. Tridimensional (top) and bi-dimensional (bottom) imaging of the longitudinal *en face* preparation of the carotid artery is depicted. (**C**) Tridimensional

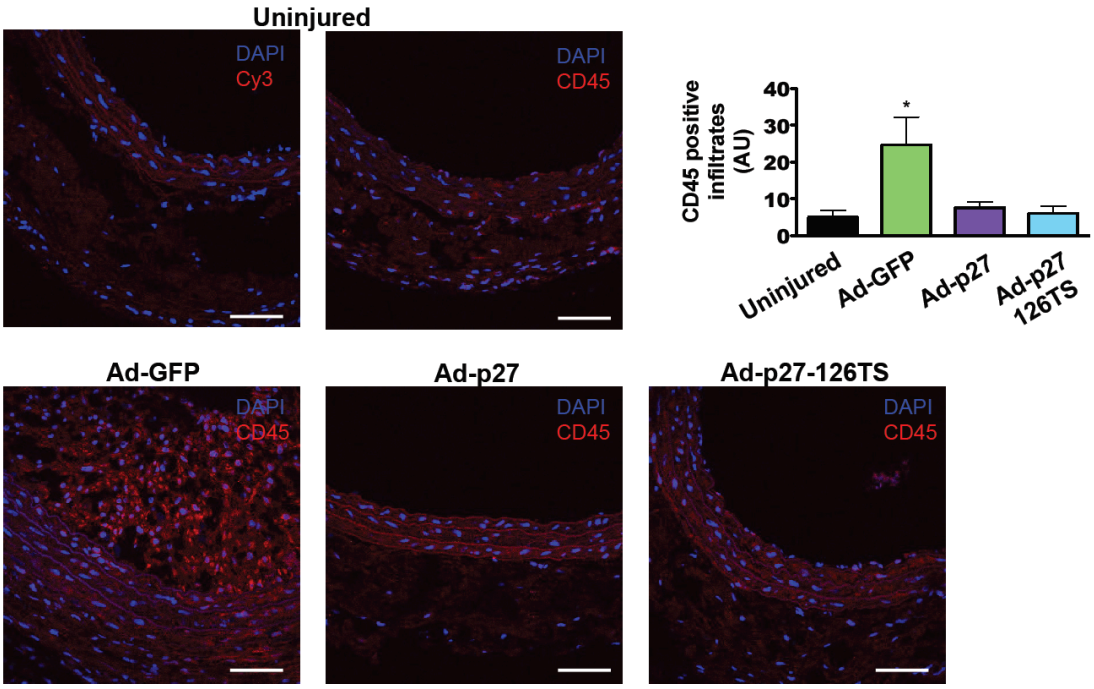
imaging (top) of a representative central portion of the vessel, with the respective 2D pictures (bottom) showing the immunostaining for Cy3, DAPI and merge, as indicated. See the **supplementary video 15 and 16** relative to the 3D images of the samples. Scale bar: 400 μm.

Figure S8



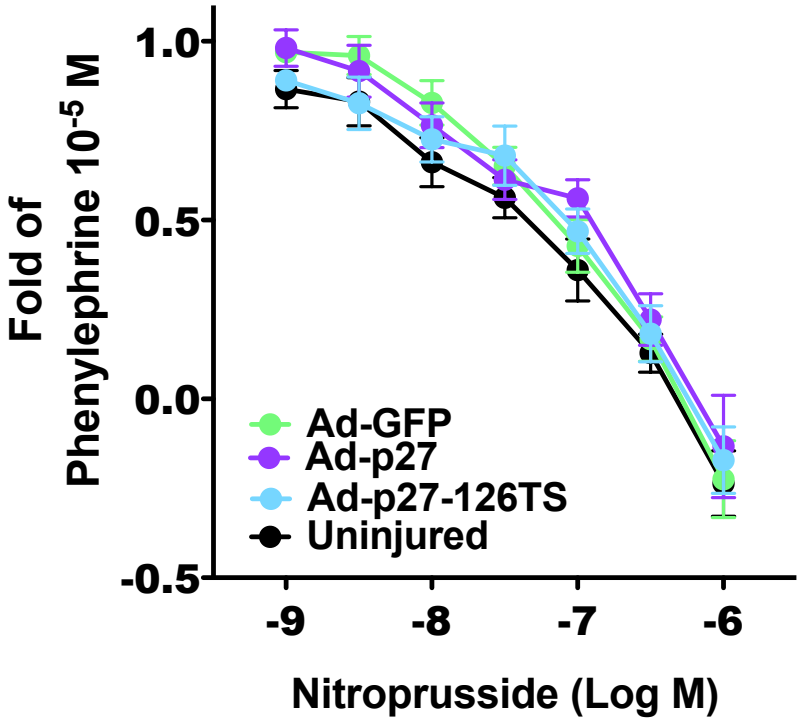
Ad-p27-126TS restores re-endothelialization in cross sections of injured carotid arteries.
(**A**) Representative sections of rat carotid arteries immunostained for the specific EC marker VE-Cadherin 4 weeks after injury. Nuclei were counterstained with DAPI. Orange scale bars: 100 μm (magnification 60×, inlays show high magnification images), arrowheads indicate EC beyond the inner autofluorescent elastic laminae. (**B**) Quantification of endothelial coverage was performed by counting the number of VE-cadherin positive cells in the circumference lumen from 6 sections/group. All data shown are means ± SEM. Data comparison was performed using one-way ANOVA, Tukey-Kramer *post hoc* test.

Figure S9



Ad-p27 and Ad-p27-126TS inhibit arterial inflammation 2 weeks after balloon injury.
Representative sections of rat carotid arteries immunostained for CD45, 2 weeks after injury. Nuclei were counterstained with DAPI. No positive staining was observed in the negative control sections (Cy3 alone). White scale bars: 100 μm (magnification 40×). CD45 positive cells were quantified by counting the number of CD45 positive cells in the intimal and medial areas from at least 3 sections/group. Data comparison was made using one way ANOVA, Tukey-Kramer *post hoc* test; **P*<0.05 versus uninjured.

Figure S10



Vasodilatory response of carotid arteries to nitroprusside.
Carotid rings 2 weeks after injury showing the vasodilatative response to nitroprusside. n=5-6 rats/group. All the data are means ± SEM.

Video Legends

Supplementary Video 1
Uninjured vessel – entire artery.
This video refers to Figure **4A**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an **uninjured** rat carotid artery immunostained with VE-cadherin (revealed by a Cy3 conjugated secondary antibody) and acquired at the confocal microscopy after assembling distinct portions of the vessel, each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 2
Uninjured vessel – central segment.
This video refers to Figure **4B**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* preparation of an **uninjured** rat carotid artery. The EC-specific immunostaining for VE-cadherin of a central portion of the vessel is shown in red (Cy3), followed by DAPI staining and then the merge (Cy3/DAPI).

Supplementary Video 3
Ad-GFP infected rat carotid artery two weeks after injury – entire artery.
This video refers to Figure **4C**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an **Ad-GFP** infected rat carotid artery **two weeks** after injury immunostained with VE-cadherin (revealed by a Cy3 conjugated secondary antibody) and acquired at the confocal microscopy after assembling distinct portions of the vessel, each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 4
Ad-GFP infected rat carotid artery two weeks after injury – central segment.
This video refers to Figure **4D**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* of an **Ad-GFP** infected rat carotid artery **two weeks** after injury. The EC-specific immunostaining for VE-cadherin of a central portion of the vessel is shown in red (Cy3), followed by DAPI staining and then the merge (Cy3/DAPI).

Supplementary Video 5
Ad-p27 infected rat carotid artery two weeks after injury – entire artery.
This video refers to Figure **4E**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an **Ad-p27** infected rat carotid artery **two weeks** after injury immunostained with VE-cadherin (revealed by a Cy3 conjugated secondary antibody) and acquired at the confocal microscopy after assembling distinct portions of the vessel, each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 6
Ad-p27 infected rat carotid artery two weeks after injury – central segment.
This video refers to Figure **4F**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* preparation of an **Ad-p27** infected rat carotid artery **two weeks** after injury. The EC-specific immunostaining for VE-cadherin of a central portion of the vessel is shown in red (Cy3), followed by DAPI staining and then the merge (Cy3/DAPI).

Supplementary Video 7
Ad-p27-126TS infected rat carotid artery two weeks after injury – entire artery.
This video refers to Figure **4 G**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an **Ad-p27-126TS** infected rat carotid artery **two weeks** after injury immunostained with VE-cadherin (revealed by a Cy3 conjugated secondary antibody) and acquired at the confocal microscopy after assembling distinct portions of the vessel [the acquisition of the central portion of the artery is shown in **H**] each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 8
Ad-p27-126TS infected rat carotid artery two weeks after injury – central segment.
This video refers to Figure **4H**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* preparation of an **Ad-p27-126TS** infected rat carotid artery **two weeks** after injury. The EC-specific immunostaining for VE-cadherin of a central portion of the vessel is shown in red (Cy3), followed by DAPI staining and then the merge (Cy3/DAPI).

Supplementary Video 9
Ad-GFP infected rat carotid artery four weeks after injury – entire artery.
This video refers to Figure **S6A**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an **Ad-GFP** infected rat carotid artery **four weeks** after injury immunostained with VE-cadherin (revealed by a Cy3 conjugated secondary antibody) and acquired at the confocal microscopy after assembling distinct portions of the vessel, each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 10
Ad-GFP infected rat carotid artery four weeks after injury – central segment.
This video refers to Figure **S6B**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* preparation of an **Ad-GFP** infected rat carotid artery **four weeks** after injury. The EC-specific immunostaining for VE-cadherin of a central portion of the vessel is shown in red (Cy3), followed by DAPI staining and then the merge (Cy3/DAPI).

Supplementary Video 11
Ad-p27 infected rat carotid artery four weeks after injury – entire artery.
This video refers to Figure **S6C**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an **Ad-p27** infected rat carotid artery **four weeks** after injury immunostained with VE-cadherin (revealed by a Cy3 conjugated secondary antibody) and acquired at the confocal microscopy after assembling distinct portions of the vessel, each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 12
Ad-p27 infected rat carotid artery four weeks after injury – central segment.
This video refers to Figure **S6D**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* preparation of an **Ad-p27** infected rat carotid artery **four weeks** after injury. The EC-specific immunostaining for VE-cadherin of a central portion of the vessel is shown in red (Cy3), followed by DAPI staining and then the merge (Cy3/DAPI).

Supplementary Video 13
Ad-p27-126TS infected rat carotid artery four weeks after injury – entire artery.
This video refers to Figure **S6E**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an **Ad-p27-126TS** infected rat carotid artery **four weeks** after injury immunostained with VE-cadherin (revealed by a Cy3 conjugated secondary antibody) and acquired at the confocal microscopy after assembling distinct portions of the vessel, each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 14
Ad-p27-126TS infected rat carotid artery four weeks after injury – central segment.
This video refers to Figure **S6F**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* preparation of an **Ad-p27-126TS** infected rat carotid artery **four weeks** after injury. The EC-specific immunostaining for VE-cadherin of a central portion of the vessel is shown in red (Cy3), followed by DAPI staining and then the merge (Cy3/DAPI).

Supplementary Video 15
Negative control (secondary antibody alone) – entire artery.
This video refers to Figure **S7B**. Representative tridimensional reconstruction of a longitudinal *en face* preparation of an uninjured rat carotid artery (stained with a Cy3 conjugated secondary antibody alone, as **negative control**) and acquired at the confocal microscopy after assembling distinct portions of the vessel [the acquisition of the central portion of the artery is shown in **C**] each obtained from a high resolution focal stack of 2D images using sequential refocusing.

Supplementary Video 16
Negative control (secondary antibody alone) – central segment.
This video refers to Figure **S7C**. Representative tridimensional reconstruction of the central portion of a longitudinal *en face* preparation of an uninjured rat carotid artery, stained with a Cy3 conjugated secondary antibody alone (as **negative control**). Cy3 staining, followed by DAPI and merge (Cy3/DAPI) are shown.