SUPPLEMENTAL MATERIAL

Supplemental Methods

Quantification of recombinant SN m-RNA expression levels in ischemic hearts

Quantitative real-time PCR was used to determine m-RNA of recombinant, plasmid derived SN content in ischemic myocardium (n=4) harvested 7, 14 and 28 days after plasmid delivery to the ischemic myocardium. Tissues were homogenized with an Ultra-Turrax® T25. Total cellular RNA was extracted using RNA-bee (amsbio). 1 µg of total RNA was transcribed using Superscript TM First-Strand Synthesis System (Invitrogen). cDNA was finally used as template for real time PCR using a BioRad C 1000 cycler with CFX96 optical reaction module and MESA GREEN qPCR Mastermix Plus for SYBR Assay (Eurogentec). For amplification, primer pairs specific for recombinant, plasmid derived SN not detecting endogenous SN m-RNA were used. Therefore, the forward primer was designed upstream of the SN sequence (forward primer: CCC AGC CGG CCA CAA; reversed primer: CCT GTC AGT TTC CCC AGC TC). The reaction sequence included denaturation for 10 min at 95°C before 40 cycles of denaturation for 15 sec at 95°C, annealing and extension for 30 sec at 61°C. Relative gene expression was calculated using the Ct method with normalization to GAPDH (forward primer: TCC TGG GCT ACA CTG AGG AC; reversed primer: GAG GGC CTC TCT CTT GCT CT).

Rat model of myocardial infarction

Briefly, Sprague-Dawley rats (Charles River Laboratories, Germany) weighing 250 to 280 g were anesthetized by intraperitoneal administration of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (5 mg/kg). Rats were endotracheally intubated and mechanically ventilated. The heart was exposed through thoracotomy at the fourth intercostal space. Myocardial infarction was induced by LAD ligation with a 7-0 polypropylene suture at the level of the pulmonary artery. Animals were randomized in a blinded fashion to one of 2 experimental groups: control group (receiving the empty plasmid

vector p-CTR) and SN-plasmid (p-SN) group. 10 min after LAD ligation, 5 depots of a total of 100 µl p-CTR (1 µg/µl) or 100 µl p-SN (1 µg/µl) were injected intramyocardially into the left ventricle. For sham operation the same procedure except LAD ligation was performed and 100 µl saline was injected.

Morphological analysis

Body-, heart- and lung-weight were determined after hemodynamic measurements.

Ischemia-reperfusion model

Ischemia-reperfusion model was generated as described previously,[1] with some modification. Rats were anesthetized as for LAD ligation and ventilated. Left-side thoracotomy was performed to reach the heart. The LAD was occluded with 8-0 silk by help of a sterile PE plastic tubing. Ischemia was evident by discoloration of the left ventricle. After 30 minutes occlusion, the ligature was loosened, and reperfusion was confirmed visually by the rapid restoration of blood flow accompanied by a change in the appearance of the ischemic myocardium from pale to red. After 3 days of reperfusion, hearts were harvested and fixed with 10% (v/v) buffered formaldehyde as described next (histological analysis). After deparaffinitation and rehydration, sections were treated with proteinase K (10 µg/ml) for 15 minutes at room temperature and washed with PBS. Apoptotic cells were visualized with TUNEL kits (Roche) using alkaline phosphatase as suggested by the manufacturer.

Histological analysis

3, 14 or 28 days after LAD ligation, rats (for each treatment n=11) were sacrificed and hearts were quickly removed. Specimens were fixed in 10% (v/v) buffered formaldehyde, dehydrated with graded ethanol series and embedded in paraffin. Alternatively, fresh tissue was embedded in OCT compound (TISSUE-TEK®, Sakura Finetek) and snap-frozen in liquid nitrogen. Serial transverse sections of 5 µm were cut across the entire long axis of the heart and subsequently mounted on slides.

Capillary- and arteriole-density

For immunofluorescence staining, frozen sections were treated with RECA-1 (abcam 9774, dilution 1:30) and alexa fluor 594 goat anti mouse (Invitrogen, A11032) in a dilution of 1:200, for assessment of capillary density. Arterioles were visualized with alpha smooth muscle actin (abcam 5694, dilution 1:20) and alexa fluor 488 goat anti mouse (Invitrogen, A11029, dilution 1:200). Capillary- and arteriole-density was evaluated by counting the number of capillaries and arterioles in 5 random and nonrepeated fields of the muscle tissue section (n=10 for each treatment).

SN and VEGFR2 in vivo staining

For SN in vivo staining, frozen sections were incubated with rabbit polyclonal SN antibody (kindly donated by Prof. Fischer-Colbrie) in a dilution of 1:100 and alexa fluor 488 goat anti rabbit (Invitrogen, A11008, dilution 1:200). Stained sections were covered with fluorescent mounting medium (Dako) containing H33258 for nuclei visualisation (1:1000).

For phospho-VEGFR2 staining, frozen sections were treated with rabbit polyclonal p-Flk-1 (Santa cruz 101821) in a dilution of 1:20 and vectastain kit (Vector, PK-6101) as suggested by the manufacturer.

Evaluation of myocardial fibrosis and quantification of myocardial infarction

For analyzing collagen accumulation, Masson´s trichrome staining was performed to delineate collagen content as a percentage of the whole heart area. After staining, slides were scanned and computerized for digital image analysis. By using Image J software, fibrotic area was calculated as the sum of fibrotic area divided by the whole heart area.

Cell culture and in vitro assays

Human coronary artery endothelial cells (HCAECs), human coronary artery smooth muscle cells (HCASMC), human cardiac fibroblasts (HCF) and human cardiac myocytes (HCM) were purchased from Promo Cell. HCAECs were cultured in EGM-2 Medium (Lonza)

containing 4.76 % (v/v) Fetal calf serum and EGM-2 Single Quots (Clonetics, Lonza). HCASMC, HCF and HCM were cultured as suggested by the manufacturer in the corresponding medium. For all in vitro assays, cells were incubated with medium without supplements containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich).

Cell migration assays were performed in a modified Boyden chemotaxis chamber in which an 8 µm-pore sized cellulose nitrate filter (Sartorius) separates the upper and the lower chamber.[2, 3] Cells were placed into the upper compartment of the chemotaxis chamber and were allowed to migrate toward SN 1, 10 and 100 ng/ml (with or without SN-Ab 1:500, VEGF-Ab (Sigma-Aldrich) 1:500, or PD98.059 10 µM (Sigma Aldrich)) placed in the lower chamber. Migration into the filter was quantified by measuring microscopically the distance from the surface of the filter to the leading front of cells. Data are expressed as chemotaxic index, which is the ratio between the distance of migration towards test attractants and that toward control medium into the nitrocellulose filter.

For tube-formation assays, an in-vitro angiogenesis kit from Chemicon was used. Cells were incubated on matrigel with SN (100 pg/ml; 1, 10 and 100 ng/ml) with or without SN-Ab, VEGF-Ab (1:500, respectively), PD98.059 or SU1498 10 µM and 40 µM (Calbiochem) and other test substances as indicated in the corresponding figures for 6 hours. VEGF (100 ng/ml) served as positive control. Capillary tubes were counted as described previously.[4]

For apoptosis assay, HCAECs were incubated with EGM-2 medium without supplements containing SN 100 ng/ml or VEGF 50 ng/ml with or without SN-Ab or VEGF-Ab (1:500, respectively) for 24 hours (HCAEC). TUNEL assay was performed according to the manufacturer's instructions (Roche) and cells positive fur TUNEL staining and for DAPI staining were counted. Results are expressed as % TUNEL positive cells of all DAPI stained cells.

Caspase 3/7 assays were performed with cells seeded in 96 well plates, as described by the manufacturer (Promega), after 16 hours treatment with the corresponding substrates.

Proliferation assays were performed using a BrdU cell proliferation ELISA kit from Roche and cells were treated as recommended by the manufacturer and analyzed after 16 hours treatment with the corresponding substrates.

Western Blotting

HCAECs were maintained as described above, plated on 60 mm tissue culture dishes and starved with EGM-2 medium without supplements containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) for 16 hours. The cells were stimulated with SN 100 ng/ml (with or without VEGF-Ab 1:500 or SU1498 40μM for different time periods. For investigation of the role of heparan sulfate proteoglycans, cells were preincubated with a mixture of heparinase I and III (both Sigma-Aldrich), 50 mU each, for 4 hours prior to stimulation with SN. Cells were lyzed with TAT buffer containing 1% Triton X-100, protease inhibitor cocktail (complete, Mini, EDTA-free, Roche) and phosphatase inhibitors (Halt™ phosphatase inhibitor cocktail, Roche). Respectively 12 μg protein (determined with bicinchoninic acid, BCA from Thermo Scientific), was separated by gel-electrophoresis (Bio-Rad, 4–15% Tris–HCl Ready Gels), transferred to Protran Nitrocellulose Transfer membranes (Whatman) and blocked by 5% non-fat milk in PBST (Phosphate-buffered saline with 0,1% Tween 20). Blots were incubated with primary antibodies against phospho-p44/42 MAPK, phospho-Akt (Ser473) and tubulin (all from Cell signaling) diluted 1: 1000 in 5% BSA in PBST for 16 hours. For receptor blotting, p-Flk-1 (Tyr 951) and Flk-1 (C-1158) antibodies (both from Santa Cruz) were allowed to incubate on the membranes over night in 5% BSA in PBST. After washing, blots were incubated for 80 minutes with the respective secondary HRP-conjugated antibodies (goat anti-rabbit and goat anti-mouse from Jackson ImmunoResearch diluted 1:10.000) and washed again. Signals were visualized by ECL staining (GE Healthcare).

RTK Profiler and Immunoprecipitation

Human phospho-RTK array Kits (R&D, ARY001) were used for investigation of receptors involved in HCAECs and HCASMCs signaling with SN. After stimulation with SN 100 ng/ml ±

VEGF-Ab for 40 minutes, cells (with or without starvation for 16 hours in empty medium) were treated as recommended by the manufacturer and a total of 300 µg protein was used. For Immunoprecipitation (IP), cells were stimulated with SN 100 ng/ml for different time periods and lyzed as described above. IP was performed by incubation of the lysates with VEGFR2 (55B11, Cell Signaling) coupled on G-sepharose (GE Healthcare) for 16 hours. After gel-electrophoresis (Bio-Rad, 5% Ready Gels), proteins were blotted to PVDF membranes (GE Healthcare) and incubated with phospho-tyrosine antibody (4G10, Upstate) for 16 hours. Total receptor was detected with Flk-1 rabbit polyclonal antibody diluted 1: 200 (C-1158, Santa Cruz). Signals were visualized by ECL staining (GE Healthcare).

I125VEGF-binding assays on HCAECs

For binding assays HCAECs were cultured in 24-well plates to near confluence and washed twice with ice cold PBS. Cells were then allowed to incubate with 250.000 cpm I125VEGF (10 ng/ml I125VEGF) ± SN (1 to 100 ng/ml) at 4°C for 2 hours in EBM-2 without supplements, containing 0.1% gelatin (G1393, Sigma-Aldrich). Thereafter, cells were washed 3 times with ice cold PBS, containing 1% BSA (Cohn V fraction, Sigma-Aldrich). Cells were lyzed with 500 µl cold PBS with 1% Triton X-100 and collected with cell scrapers (Greiner). 300 µl of each sample were transferred to radio-immuno tubes and measured with a γ-counter (Perkin Elmer). For saturation curves, HCAECs were incubated with increasing amounts of I125VEGF (0.1 to 20 ng I125VEGF) with or without addition of SN 100 ng/ml. To determine specific binding a 500-fold excess of cold VEGF was added. Data from saturation were analyzed using GraphPad Prism 5.0. Investigation of influence of heparinase was performed by preincubation of the cells with a mixture of heparinase I and heparinase III, 50 mU each, for 4 hours at 37°C, prior to the experiment.

I125VEGF-binding assays to heparin, neuropilin 1 and VEGF-receptor 2

Maxisorb tubes (Nunc) were coated with 100 µl heparin-BSA complex (BSA: 0.2 µg/ml Tris-EDTA buffer, pH 7.4), or BSA-control complex over night at 4°C. Heparin-BSA and BSA-

control complex were synthesized as described previously.[5] After 3 times washing with 350 µl PBST (phosphate-buffered saline with 0.1% Tween 20), tubes were blocked with 1% BSA (150 µl) for 1 hour at room temperature and washed again 3 times with PBST. Thereafter, I125VEGF 50.000 cpm (2.5 ng/ml I125VEGF) in 50 µl phosphate buffer (10 mM, pH 7.0) containing 1 % BSA and 10 to 100 ng/ml SN, was added and allowed to incubate for 2 hours at room temperature. After 3 times washing with PBST, tubes were counted in a γ-counter. For receptor binding assays, tubes were incubated with 1 µg/ml carrier free, recombinant human neuropilin 1, VEGF-receptor 2 (both R&D) or BSA as control over night at 4°C and then treated as specified above. To determine specific binding, a 500-fold excess of cold VEGF was added.

Real time PCR of SG2

Quantitative real-time PCR was used to determine m-RNA of SG2 levels in HCM, HCAEC and HCF (Promo Cell) after 72 hours in the corresponding culture medium (Promo Cell) under normoxic or hypoxic conditions. Normal humidified tissue culture incubators with 5% CO_2 were used for the normoxic cultures. For decreased oxygen cultures, plates were inserted into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA, USA), which were flushed with a custom gas mixture containing 5% CO_2 and 95% N_2 for 15 minutes daily. RNA was isolated with RNeasy Mini Kits (Qiagen) as suggested by the manufacturer. 1 µg of total RNA was transcribed as described above and cDNA was finally used as template for real time PCR using a BioRad C 1000 cycler with CFX96 optical reaction module and SsoFast™ Eva Green® Supermix (BioRad). For amplification, primer pairs specific for human SG2 were used (forward primer: AGA ACG GGG AGG AAT ATG CT; reversed primer: GGT CTT TGC TTC AGC CAT GT). The reaction sequence included 35 sec at 95°C and 10 sec at 60°C for 40 cycles. Relative gene expression was calculated using the Ct method with normalization to tubulin (forward primer: CAG GCT GGT GTC CAG ATT GGC AA; reversed primer: CGT CTC ACT GAA GAA GGT GTT GAA GGA).

Radioimmunoassay (RIA) for SN

RIA was performed as described previously.[6]

Supplemental Results

SN gene therapy reduces organ congestion 4 weeks after MI

After SN-plasmid injection, heart-weight/body-weight ratio (HW/BW) and lung-weight/body-weight (LW/BW) were significantly lower than in the control plasmid group 4 weeks after MI (suppl. Table. 1).

SN gene therapy reduces apoptosis in a rat model of myocardial ischemia reperfusion

TUNEL staining (with alkaline phosphatase) for apoptotic cells 3 days after 30 minutes of ischemia showed significantly lower apoptotic nuclei in the SN-treated animal group compared to control group (p-SN: 34.9±1.4 vs. p-CTR: 39.5±1.2, #p=0.02; n=3; suppl. Fig. 1).

SN induces migration of HCASMCs

SN caused induction of chemotaxis in HCASMCs in concentrations of 10 and 100 ng/ml, with similar effects (SN 10 ng/ml relative CI: 1.75±0.13, SN 100 ng/ml 1.74±0.22, p=0.0002 vs. Control). SN mediated migration was comparable to the effects of VEGF 50 ng/ml (rel. CI: 1.89±0.19) and PDGF-BB 10 ng/ml (rel. CI: 1.91±0.13). SN induced migration could not be blocked with VEGF-Ab (rel. CI: 1.63±0.41; suppl. Fig. 2A).

SN mediated tube formation is not blocked by bFGF-Ab in HCAECs

SN 100 ng/ml induced angiogenesis in a matrigel assay with HCAECs (relative capillary tube formation 2.35±0.19, p=0.00005 vs. Control; suppl. Fig. 2B). Addition of VEGF-Ab abolished SN-induced tube formation (SN + VEGF-Ab 1:500, 1.24±0.13, p=0.0004 vs. SN and SN + VEGF-Ab 1:1000, 1.38±0.31, p=0.02 vs. SN). Interestingly, a bFGF-Ab did not influence SN-induced tube formation (SN + bFGF-Ab 1:500, 2.15±0.22, p=0.002 vs. Control). VEGF 50 ng/ml served as positive control.

SN does not increase binding of I125VEGF to VEGFR2

In order to test the possibility that SN increases the binding of VEGF to VEGFR2, we performed binding assays with I125VEGF. Maxisorb tubes were coated with recombinant, human VEGFR2 and binding assays in the presence or absence of SN 10 and 100 ng/ml were performed. SN 100 ng/ml did not increase I125VEGF binding to VEGFR2 (I125VEGF bound to VEGFR2: 745.6±15.2 fmol/µg; I125VEGF + SN 100 ng/ml bound to VEGFR2: 780.3±5.6 fmol/µg; suppl. Fig. 3A).

SN increases binding of I125VEGF to heparin

For heparin binding studies, heparin-coated beads were incubated with I125VEGF in presence or absence of SN 100 ng/ml. SN significantly increased the binding of I125VEGF to heparin (I125VEGF 2090.5±90.2 fmol vs. I125VEGF + SN 3177.6±52.7 fmol, p=0.009; suppl. Fig. 3B).

SN shows a tendency to improve cardiac function and blood vessel density 2 weeks after MI

Echocardiographic assessment of myocardial function 2 weeks after LAD ligation shows a tendency to improve left ventricular ejection fraction (LVEF: p-SN 54.3±7.3 % vs. p-CTR 38.8±3.9 %; sham operation 59.7±4.2 %) and fractional shortening (LVFS: p-SN 34.2±3.7 % vs. p-CTR 24.4±3.7 %; sham operation 42.9±2.6 %) in animals treated with SN-plasmid (p-SN) compared to control plasmid (p-CTR). Results did not reach statistical significance (suppl. Fig. 4A). Left ventricular end diastolic diameter (LVEDD: p-SN 7.8±0.4 mm vs. p-CTR 8.5±0.5 mm;) and systolic diameter (LVESD: p-SN 5.2±0.5 mm vs. p-CTR 6.4±0.5 mm) also revealed a trend for inhibition of left ventricular dilatation 2 weeks after MI by SN gene therapy compared to treatment with control vector. Results did not reach statistical significance (suppl. Fig. 4B). Quantification of RECA-positive capillaries (p-SN: 292.8±56.9 vs. p-CTR: 266.3±62.5, n=5) and α−SMA-positive arteries/arterioles (p-SN: 5.4±1.4 vs. p-

CTR: 4.6±1.2, n=5) in the rat myocardium border zone of the MI, 2 weeks after LAD ligation and treatment with SN-plasmid or control plasmid again showed a trend to improve with p-SN but did not reach statistical significance between groups (suppl. Fig. 4C).

Hypoxia increases SN in human cardiac myocytes (HCM) but not in HCAECs in vitro

SG 2 m-RNA is up-regulated under hypoxia in HCMs after 72 hours as measured by real time PCR (Hypoxia: 1.48±0.05, p=0.002 vs. Normoxia, suppl. Fig. 5A). SN is also increased at the protein level by hypoxia, as detected by radioimmunoassay (Hypoxia 57.2±1.6 fmol/10^5 cells vs. Normoxia: 39.3±1.1 fmol/10^5 cells, p=0.0009, suppl. Fig. 5B). In contrast to HCM, SG2 mRNA levels are not influenced by hypoxia in HCAECs (Hypoxia: 0.96±0.3, suppl. Fig. 5C).

Hypoxia increases SN in rat cardiac myocytes in vivo

Secretogranin 2 (SG2, pro-hormone of SN) m-RNA is up-regulated in ischemic rat heart in vivo 3 days after MI as measured by real time PCR (ischemic ventricle: 2.2±0.3 vs. CTR, p=0.009; n=3). Healthy heart tissue served as control (suppl. Fig. 6A). Immunofluorescent staining for SN (green) in the border zone of MI in rat hearts 3 days after LAD ligation confirms findings of real time PCR. H33258 was used for nuclei visualization (suppl. Fig. 6B).

Hypoxia does not increase SG2 mRNA in HCF

m-RNA of human cardiac fibroblasts (HCF) after 72 hours hypoxia was screened for Secretogranin 2 (pro-hormone of SN) by real time PCR (Hypoxia: 0.90±0.07; n=5), but did not reveal elevated SG2 m-RNA levels under hypoxic conditions (suppl. Fig. 7A).

SN does not increase proliferation in HCF

BrdU assays performed with HCF showed no SN-induced HCF proliferation (SN 10 ng/ml: 1.13±0.04; SN 100 ng/ml: 1.18±0.05). bFGF served as positive control (3.01±0.07; *p=0.00003 bFGF vs. Control, n=4). Results are shown as mean ± SEM (suppl. Fig. 7B).

SN does not inhibit apoptosis in HCF

SN showed no anti-apoptotic effects on HCF as measured by caspase 3/7 assays after 16 hours of starvation (SN 10 ng/ml: 0.96±0.02; SN 100 ng/ml: 0.97±0.06). bFGF was used as positive control (0.81±0.03; #p=0.047 bFGF vs. Control, n=4). Results are shown as mean ± SEM (suppl. Fig. 7C).

SN induced tube formation in HUVECs is not influenced by bFGF-Ab or VEGF-Ab. Tube formation assays with SN 100 ng/ml in HUVECs revealed no influence of bFGF-Ab or VEGF-Ab on in vitro angiogenesis in these cells (*p<0.001 vs. Control; suppl. Fig. 8).

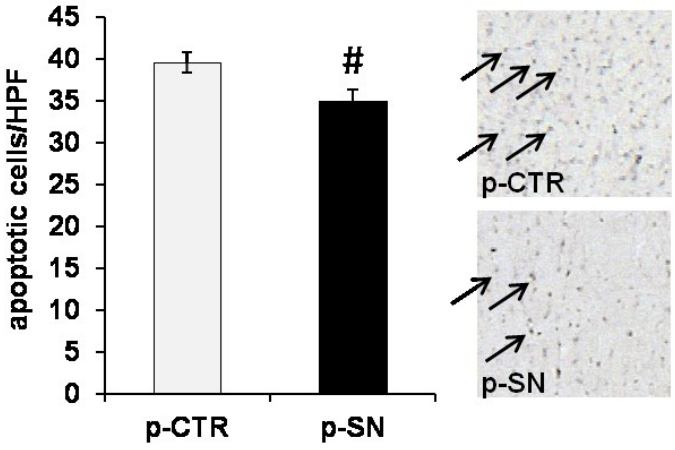
Supplemental Table

Table 1

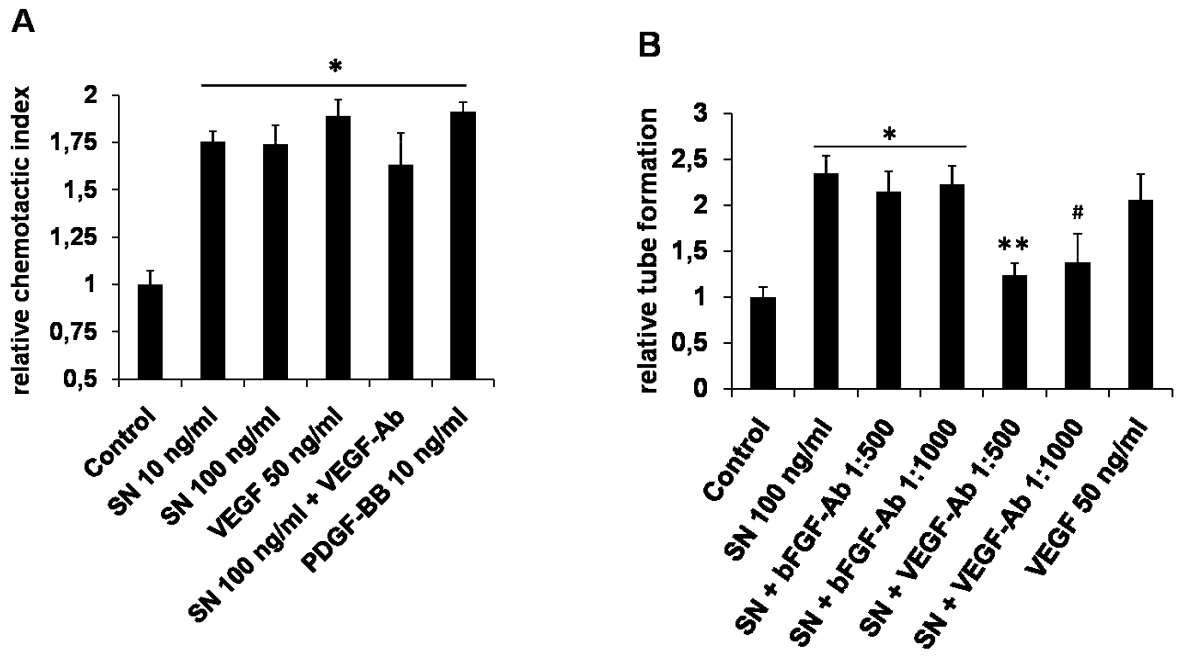
	HW/BW [mg/g]	LW/BW [mg/g]
p-CTR	3.66 ± 0.10	5.10 ±0.20
p-SN	3.23 ± 0.09	4.54 ± 0.20
	p<0.005	p<0.05

Supplemental Figures

Supp. Figure 1



Supp. Figure 2



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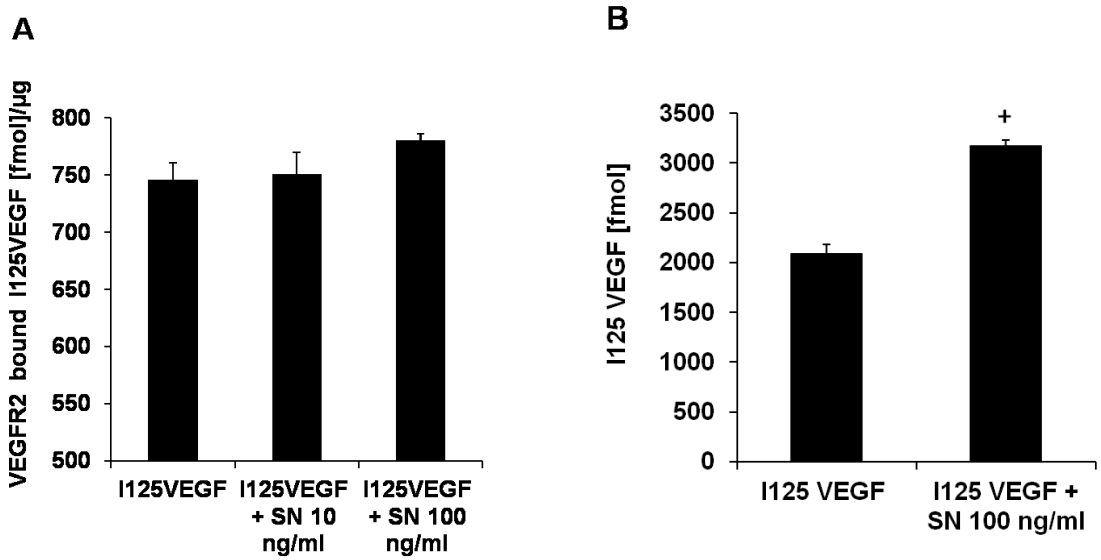
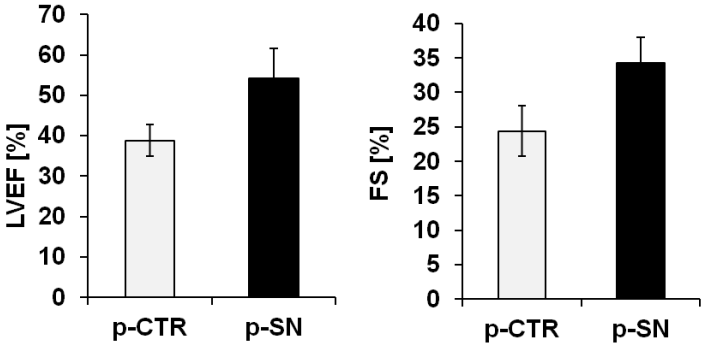


Supp. Figure 4

A



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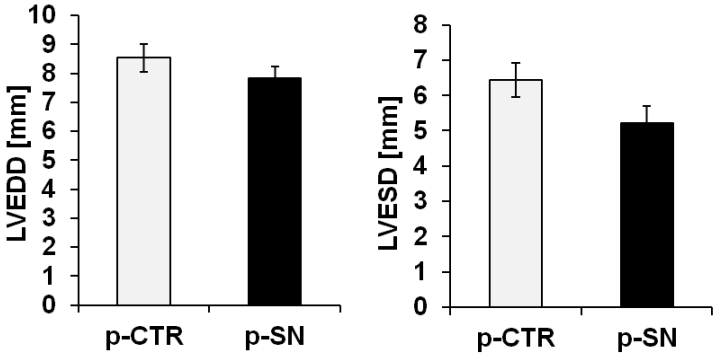

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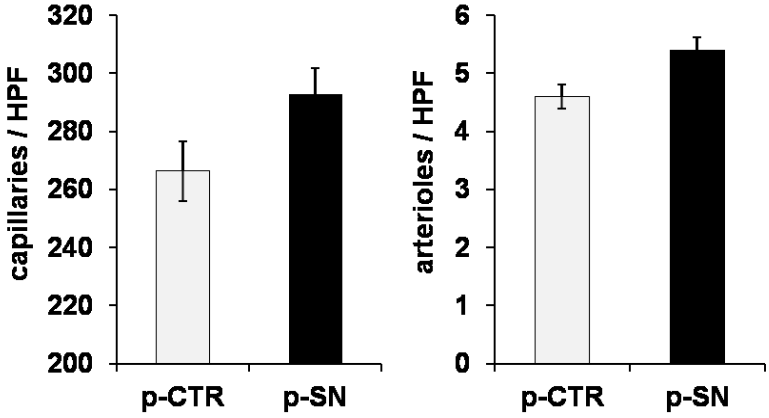
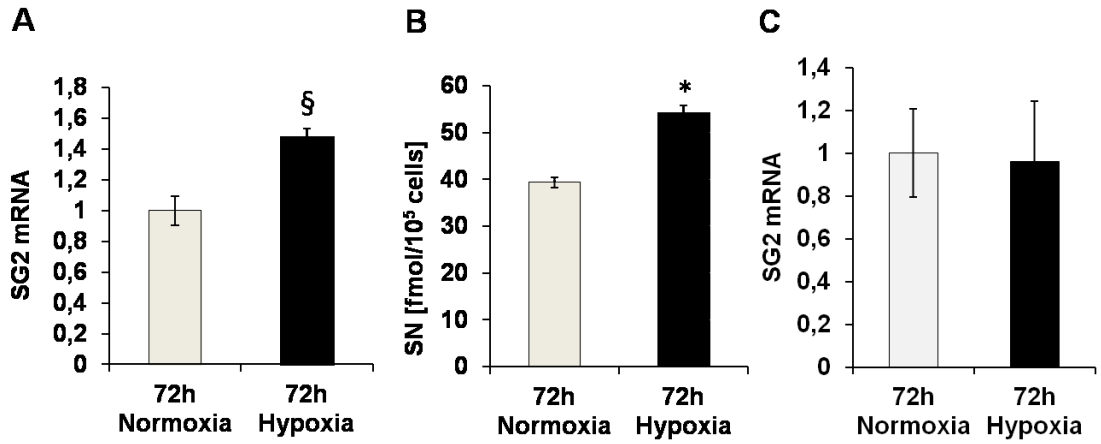


Suppl. Figure 5



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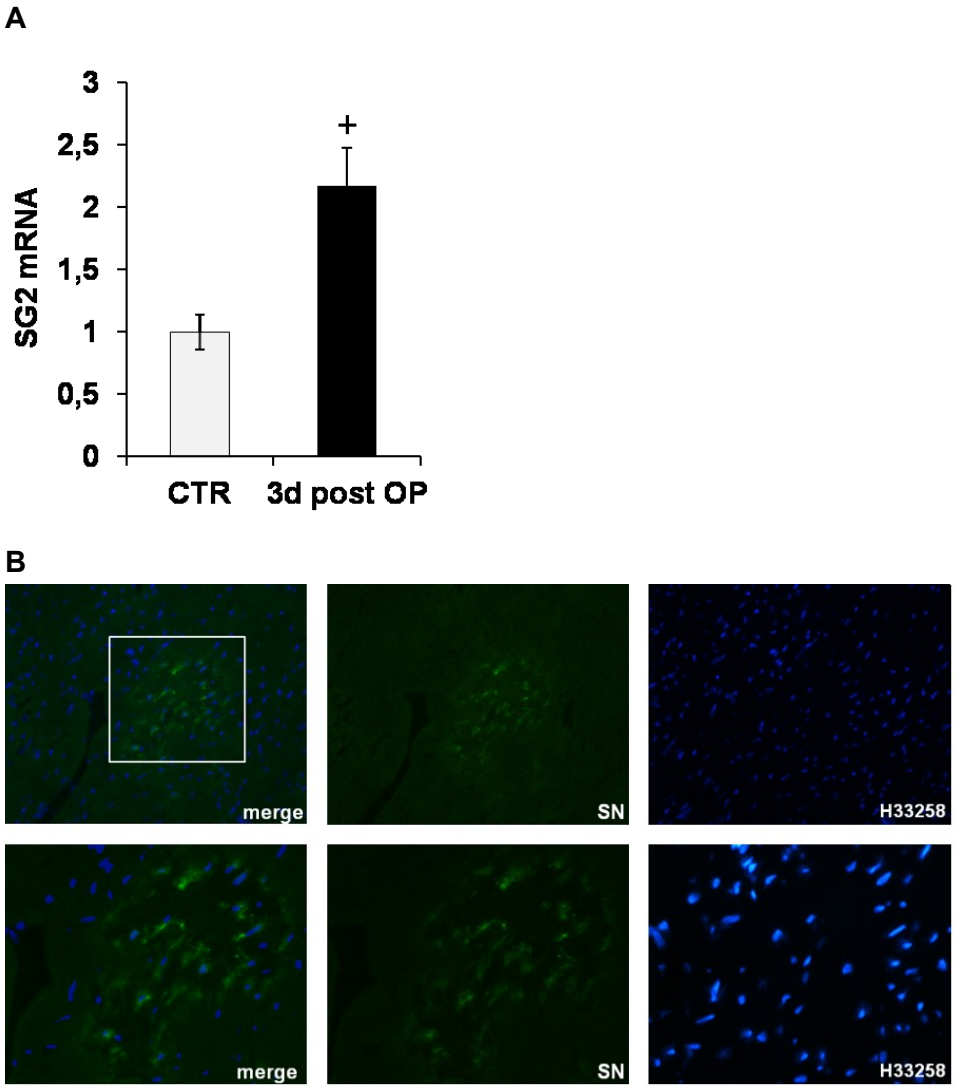
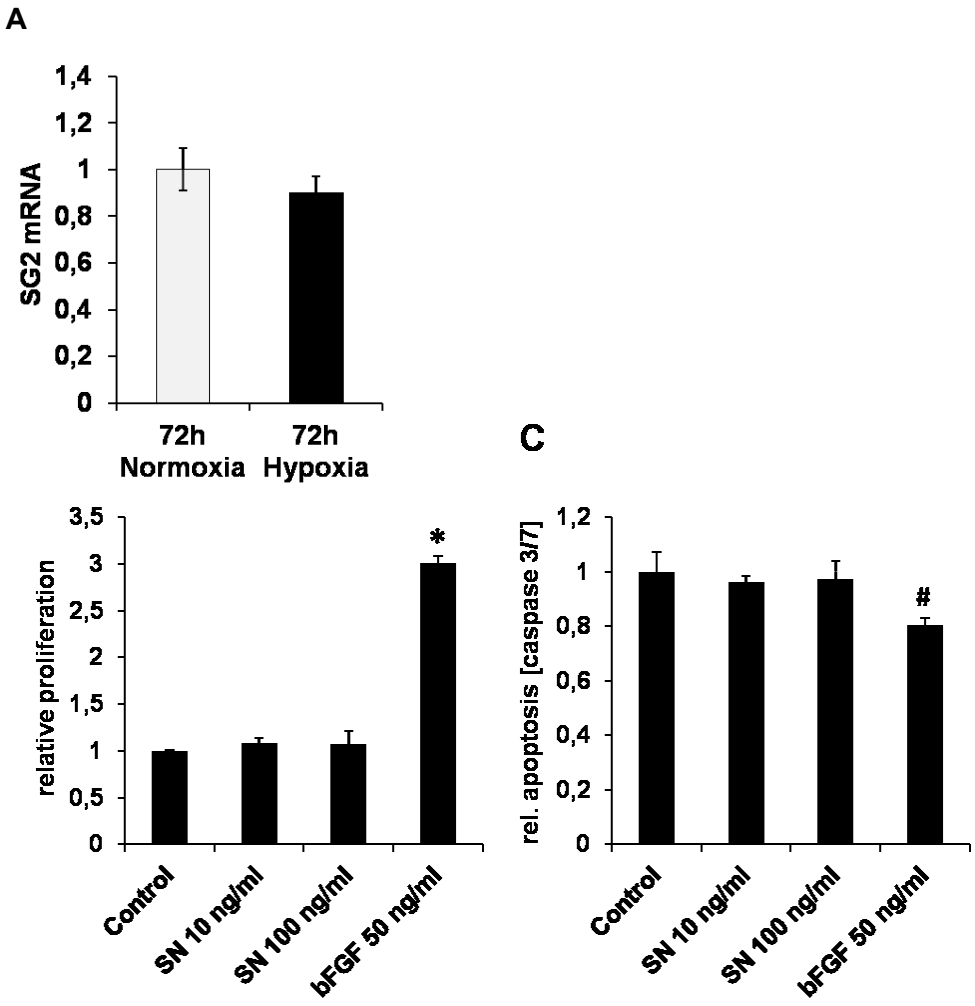
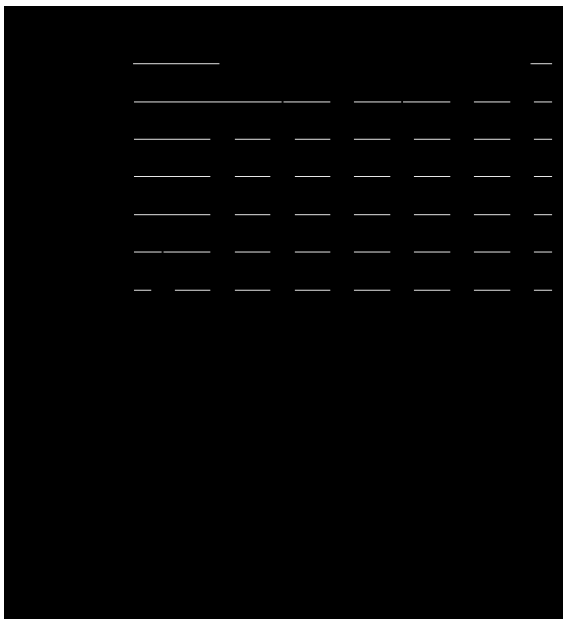


Supp. Figure 7



Supp. Figure 8



Supplemental Legends

Table 1 Morphological data at 4 weeks after MI showed a significant difference in heart-weight/body-weight ratio (HW/BW) and lung-weight/body-weight ratio (LW/BW) in the SN treated group. Results are shown as mean ± SEM (HW/BW (n=16): p=0.0042 p-SN vs. p-CTR; LW/BW (n=11): p=0.029 p-SN vs. p-CTR).

Supp. Figure 1. SN gene therapy reduces apoptosis in a rat model of ischemia reperfusion. TUNEL staining for apoptotic nuclei 3 days after 30 min ischemia (arrows indicate apoptotic (violet) nuclei). Quantification showed significant inhibition of cell apoptosis in the SN treated animal group vs. p-CTR group (#p<0.05; n=3 per group). Results are shown as mean ± SEM.

Supp. Figure 2. A SN induces migration of HCASMCs. Migration assays with HCASMCs revealed chemotaxis towards SN (10 and 100 ng/ml), VEGF 50 ng/ml and PDGF-BB 10 ng/ml. A VEGF-Ab (1:500) did not significantly influence SN-activated migration of SMCs (*p<0.001 vs. Control; n=3). **B SN mediated tube formation is not blocked by bFGF-Ab in HCAECs.** HCAECs were allowed to form tubes in a matrigel assay in presence or absence of SN 100 ng/ml ± b-FGF or VEGF-Ab. VEGF served as positive control. SN induced tube formation is blocked by VEGF-Ab but not by bFGF-Ab (*p<0.001 SN and SN + bFGF-Ab vs. Control; SN+VEGF-Ab 1:500 vs. SN, ** p<0.001; SN+VEGF-Ab 1:1000 vs. SN, # p<0.05; n=3).

Supp. Figure 3. A SN does not increase binding of I125VEGF to VEGFR2. Maxisorb tubes were coated with recombinant, human VEGFR2 and binding assays in the presence or absence of SN 10 and 100 ng/ml were performed and analyzed in a γ-counter. SN 100 ng/ml did not increase I125VEGF binding to VEGFR2 (n=4). **B SN increases binding of I125VEGF to heparin-sepharose.** Binding assays with heparin-coated sepharose beads

showed that SN 100 ng/ml significantly increased I125 VEGF binding to heparin (+p<0.01 VEGF vs.SN + VEGF; n=4). Results are shown as mean ± SEM.

Supp. Figure 4. A Effect of SN gene therapy on cardiac function 2 weeks after MI. Echocardiographic assessment of myocardial function showed a tendency to improvement of left ventricular ejection fraction and fractional shortening (LVEF and LVFS) 2 weeks after MI and treatment with SN-plasmid (p-SN) compared to control plasmid (p-CTR). Results are shown as mean ± SEM (n=9 per group) but did not reach statistical significance. **B Effects of SN gene therapy on left ventricular remodeling.** Echocardiographic analysis of left ventricular end diastolic and systolic diameter (LVEDD and LVESD) revealed a trend for inhibition of left ventricular dilatation 2 weeks after MI by SN gene therapy compared to treatment with control vector. Results are shown as mean ± SEM but did not reach statistical significance. **C Quantification of CD-31 positive capillaries and α−SMA positive arteries/arterioles** in the rat myocardium border zone of the MI, 2 weeks after LAD ligation and treatment with SN plasmid or control plasmid. Results are shown as mean ± SEM but did not reach statistical significance.

Supp. Figure 5. A Hypoxia increases SN in cardiac myocytes but not in HCAECs. Secretogranin 2 (pro-hormone of SN) m-RNA is up-regulated under hypoxia in human cardiac myocytes after 72 hours as measured by real time PCR (§p<0.005 vs. Normoxia; n=4). Results are shown as mean ± SD. **B** SN is also increased by hypoxia at the protein level as detected by radioimmunoassay (*p<0.001 vs. Normoxia; n=4). Results are shown as mean ± SEM. **C** SG2 mRNA is not increased in human arterial coronary ECs (HCAECs) after 72 hours of hypoxia. Results are shown as mean ± SD.

Suppl. Figure 6. A Hypoxia increases SN in cardiac myocytes in vivo. Secretogranin 2 (pro-hormone of SN) m-RNA is up-regulated in ischemic rat cardiac myocytes 3 days after MI as measured by real time PCR (+p<0.01 vs. CTR; n=3). Non-ischemic heart tissue served as control. Results are shown as mean ± SD. **B** Immunofluorescent staining for SN in

the border zone of MI in rat hearts 3 days after LAD ligation confirm findings of real time PCR. H33258 was used for nuclei visualization.

Supp. Figure 7. A Hypoxia does not increase SG2 mRNA in HCF. Secretogranin 2 (pro-hormone of SN) m-RNA is not influenced by hypoxia in human cardiac fibroblasts after 72 hours as measured by real time PCR (n=5). Results are shown as mean ± SD. **B SN does not increase proliferation in HCF.** bFGF served as positive control (*p<0.01 bFGF vs. Control, n=4). Results are shown as mean ± SEM. **C SN does not inhibit apoptosis in HCF.** As positive control bFGF was used (#p<0.05 bFGF vs. Control, n=4). Results are shown as mean ± SEM.

Supp. Figure 8. SN induced tube formation in HUVECs is not influenced by bFGF-Ab or VEGF-Ab. Tube formation assays with SN 100 ng/ml ± bFGF-Ab or VEGF-Ab in HUVECs (*p<0.001 vs. Control; n=3). Results are shown as mean ± SEM.

Supplemental References

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