# Natriuretic Peptide Receptor B modulates the proliferation of the cardiac cells expressing the Stem Cell Antigen-1.

Stéphanie Rignault-Clerc,[1] Christelle Bielmann,[1] Lucas Liaudet,[2] Bernard Waeber [1] François Feihl ,[1] Nathalie Rosenblatt-Velin [1*]

[1] Unité de Physiopathologie Clinique and [2] Service de Médecine Intensive Adulte, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Switzerland.

* Corresponding author

## SUPPLEMENTAL METHODS

### Cell isolation and proliferation

Neonatal mice were sacrificed by decapitation with surgical scissors and the hearts removed. Hearts (1-2 days-old) were enzymatically digested with 0.45 mg/ml collagenase II (Worthington, Biochemical Corporation, USA) and 1 mg/ml pancreatin (Sigma-Aldrich). Cardiomyocytes were separated from the non-myocyte cells (NMCs) by two differential plating. NMCs were first cultured **in the proliferation medium**: a 3:1 mixture of DMEM and Medium 199 (Invitrogen Corp, San Diego, CA, USA) supplemented with 10% horse serum (Oxoid), 5% fetal bovine serum (FBS) (Invitrogen), 10 mM Hepes, 100 U/ml penicillin G, and 100 microg/ml streptomycin.

### Western blot

Total proteins were extracted from sorted Sca-1$^+$ cells after lysis in a buffer containing Nonidet P-40 0.5%, NaCl 150 mM, Na-orthovanadate 1 mM, NaF 10 mM, Tris-HCL pH=7.5 10 mM, PMSF 1 mM, EDTA pH=8 1 mM, aprotinin 10 μg/ml, leupeptin 10 μg/ml, pepstatin 1 μg/ml. 30 μg of these total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Biorad), and blocked for 1h at room temperature in Odyssey blocking buffer (1/2 in TBS Li-COR Biosciences).

### Neonatal and infarcted mice treated with BNP

BNP (1 microg/mouse), CNP (1 microg/mouse), or NaCl was injected intraperitoneally (ip) into 3-4 days old C57BL/6 mice (American Peptide Co, Sunnyvale, CA, US) every 2 days during 2 weeks as previously described [1]. Mice were sacrificed 3 days after the

last injection using ip lethal injection of pentobarbital (150 mg/kg). The breathing arrest was controlled and cervical dislocation was performed.

In adult mice, the left anterior descending coronary artery (LAD) was occluded in 10 weeks-old C57 BL/6 mice to induce MI. Mice were anesthetized by ip injection of ketamine/xylazine/acepromazine (65 mg/kg; 15 mg/kg; 2 mg/kg). Mice were moved to a heated plateform. During surgery, the depth of mouse anesthesia was tested by gentle pinch the legs. Directly after the surgery, NaCl or BNP (1 microg/20g in 20 microl) was injected into the myocardium and thereafter BNP (2 microg/mouse) was injected ip every 2 days. After surgery, temgesic (0.1 mg/kg) was injected subcutaneously to reduce the pain, and injections were performed twice per day for up to 4 days. Mice were sacrificed 10 days after MI induction by lethal injection of pentobarbital (150 mg/kg intraperitoneally). The breathing arrest was controlled and cervical dislocation was performed.

## REFERENCE


1. Bielmann, C. *et al.* Brain natriuretic peptide is able to stimulate cardiac progenitor cell proliferation and differentiation in murine hearts after birth. *Basic Res Cardiol* **110**, 455-72 (2015).

**Supplemental Table S1.** Flow cytometric characterization of Non-Myocyte Cells (NMCs) isolated directly from murine hearts or after cell culture.

| Age of mice | strain | n | days after isolation | NPR-A$^+$ | NPR-B$^+$ | c-kit$^+$ | Sca-1$^+$ | NPR-A$^+$ /c-kit$^+$ | NPR-A$^+$ /Sca-1$^+$ | NPR-B$^+$ /c-kit$^+$ | NPR-B$^+$ /Sca-1$^+$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Adult | WT | 6 | 0 | 3.3±1.0 | 6.3±1.1 | 6.2±1.1 | 50.7±2.7 | 2.1±0.7 | 1.8±0.2 | 3.5±0.7 | 3.7±0.8 |
| Neonatal | WT | 6 | 0 | 2.9±0.3 | 5.6±1.1 | 2.9±0.3 | 15.3±1.6 | 2.9±0.3 | 2.0±0.6 | 2.7±0.4 | 4.1±1.4 |
| Neonatal | WT | 5 | 3° | 3.8±0.5* | 2.6±1.0* | 2.3±0.9 | 49.0±4.2* | 0.6±0.2* | 3.3±0.4 | 0.6±0.2* | 2.1±0.9 |
| Neonatal | NPR-A $^{-/-}$ | 3 | 3 | -- | 1.9±0.2 | 0.7±0.2 | 64.2±9 | -- | -- | 0.4±0.1 | 1.9±0.5 |
| Neonatal | NPR-B $^{-/-}$ | 6 | 3 | 2.9±0.7 | -- | 1.3±0.5 | 52.2±5.6 | 0.9±0.2 | 2.3±0.5 | -- | -- |

Data represent the percentage of cells positive for the different markers and are expressed as the mean ± SEM of the indicated number of experiments (n). Neonatal and adult non-myocyte cells (NMCs) were analysed directly after isolation (0 days after isolation) or ° 3 days after the first passage at confluence, at the onset of BNP treatment. Cells expressing NPR-A and NPR-B (C57BL/6 or WT cells) were compared to cells expressing either only NPR-B (NPR-A KO mice) or only NPR-A (NPR-B deficient mice). * $p<0.05$ between WT cells after isolation and after 3 days after the first passage.

**Supplemental Table S2.** Antibodies used in flow cytometry analysis, cell sorting, immunohistology and Western blot analysis.

### Flow Cytometry analysis and Cell sorting

| | | |
|---|---|---|
| rat-anti Sca-1 PE-Cy5 or FITC | 1/50 | eBiosciences |
| rat anti-c-kit PE-Cy5 or FITC | 1/50 | eBiosciences |
| rat anti-CD31 APC | 1/100 | eBiosciences |
| rat anti-PDGFR-$\alpha$ PE | 1/80 | eBiosciences |
| rabbit anti-NPR-A | 1/50 | Abcam |
| + donkey anti-rabbit FITC | 1/1000 | Molecular Probes |
| goat anti-NPR-B | 1/20 | Santa Cruz Biotechnology |
| + chicken anti-goat FITC | 1/500 | Molecular Probes |

### Immunohistology

| Primary antibodies | | | Secondary antibodies | | |
|---|---|---|---|---|---|
| rabbit anti-NPR-A | 1/50 | Abcam | horse anti-goat biotin | 1/200 | Vector |
| goat anti-NPR-B | 1/50 | Santa Cruz Biotechnology | horse anti-rabbit biotin | 1/200 | Vector |
| goat anti-Nkx2.5 | 1/20 | Santa Cruz Biotechnology | donkey anti-rabbit Alexa 594 | 1/500 | Molecular Probes |
| rabbit anti-Nkx2.5 | 1/100 | Abcam | donkey anti-goat Alexa 594 | 1/1000 | |
| goat anti-Troponin I | 1/100 | Santa Cruz Biotechnology | donkey anti-rat Alexa 488 | 1/1000 | |
| rat anti-Sca-1 | 1/1000 | Abcam | goat anti-rabbit Alexa 594 | 1/2000 | |
| rabbit anti-PCNA | 1/100 | Abcam | donkey anti-rabbit Alexa 488 | 1/1000 | |
| rat anti-CD31 biotin | 1/200 | Becton Dickinson | Steptavidine Alexa 594 | 1/1000 | |
| rabbit anti-von Willbrand Factor | 1/500 | Dako | Streptavidine Alexa 647 | 1/1000 | |
| Rabbit anti-smooth muscle actin | 1/200 | Abcam | chicken anti-goat Alexa 488 | 1/1000 | |

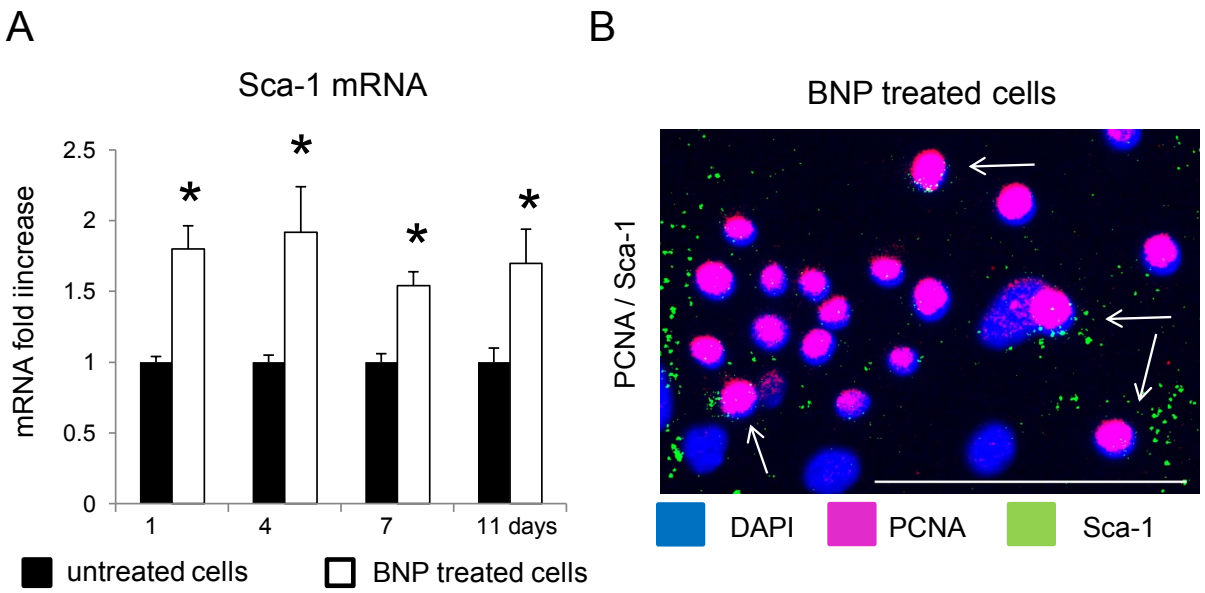
### Western blot analysis

| Primary antibodies | | | Secondary antibodies | | |
|---|---|---|---|---|---|
| rabbit anti-phospho-phospholamban | 1/1000 | Millipore | goat anti-rabbit Alexa 680 | 1/5000 | Molecular Probes |
| mouse anti-phospholamban | 1/500 | Millipore | Anti-mouse IRDye 800 | 1/5000 or 1/20000 (for tubulin) | Rockland Immunochemicals |
| rabbit anti- p38 | 1/1000 | Cell Signaling | | | |
| rabbit anti-phospho p38 | 1/500 | Cell Signaling | | | |
| Mouse anti-tubulin | 1/20 000 | Sigma | | | |

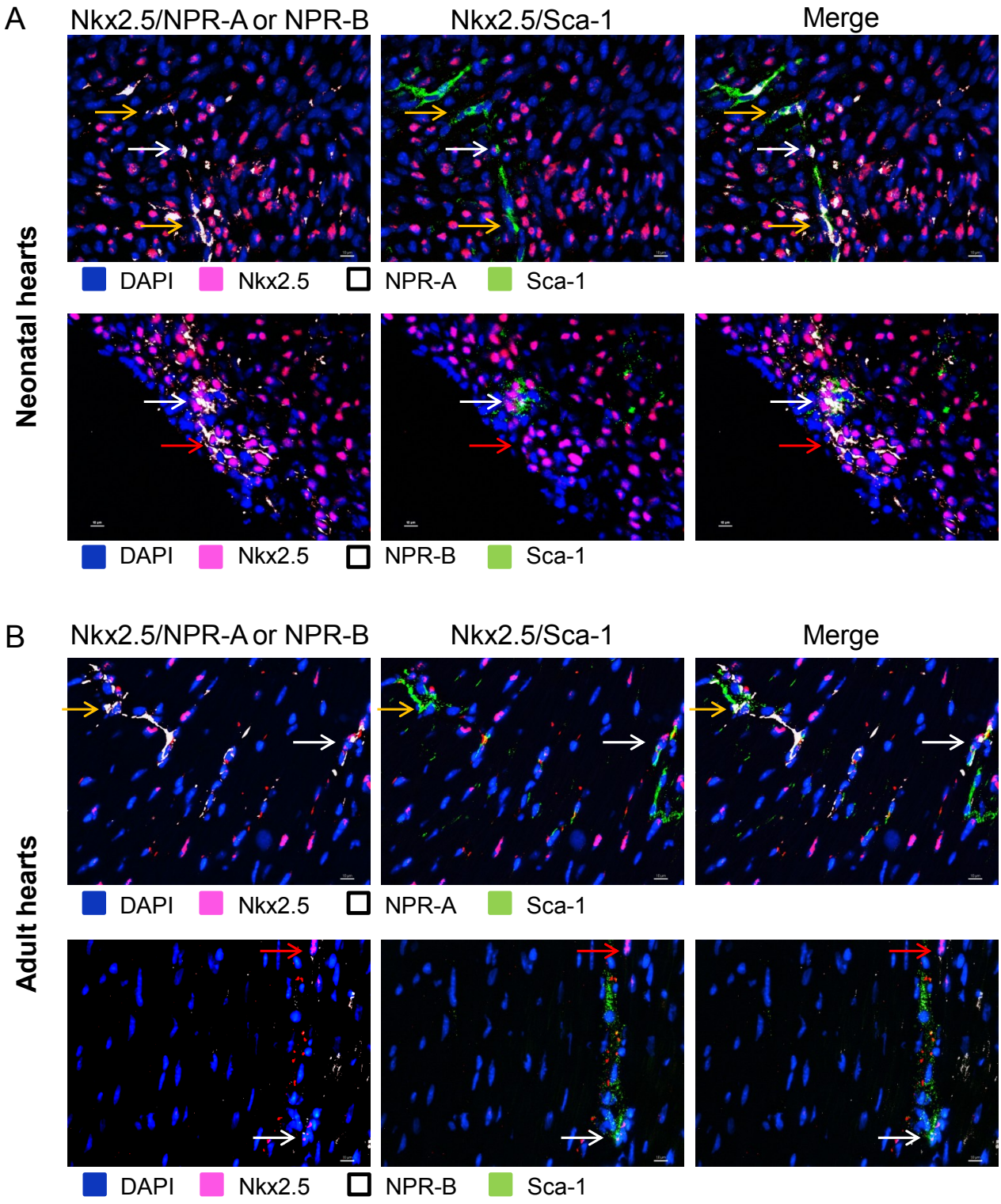
**Supplemental Table S3:** Sequences of primers used in quantitative RT-PCR.

| Gene | Sense | Anti-sense | Product size (bp) |
|---|---|---|---|
| **Brachyury** | CCTCCCTTGTTGCCTTAGAGTAGTT | GCAGATTGTCTTTGGCTACTTTGTC | 185 |
| **CD90** | TGGGTGCAGCAACTGGAGGC | CTCGGGACACCTGCAAGACTGA | 181 |
| **CD31** | GCCTCACCAAGAGAACGGAAGGC | CTGCTTTCGGTGGGGACAGGC | 158 |
| **c-kit** | ATCTGCTCTGCGTCCTGTTG | CTGATTGTGCTGGATGGATG | 108 |
| **Collagen 1α1** | AATGGCACGGCTGTGTGCGA | AACGGGTCCCCTTGGGCCTT | 183 |
| **Collagen 1α2** | GGCCCCCTGGTATGACTGGCT | CGCCACGGGGACCACGAATC | 129 |
| **DDR2** | TTCCCTGCCCAGCGAGTCCA | ACCACTGCACCCTGACTCCTCC | 181 |
| **eNOS** | GGCTGTGGTAGTTAGGGCATC | AGGTTTGGGTTGGGCATCT | 165 |
| **Gata-4** | CTGTCATCTCACTATGGGCA | CCAAGTCCGAGCAGGAATTT | 259 |
| **Hand2** | CCTTCAAGGCGGAGATCAAGA | CCTGTCCGGCCTTTGGTTTT | 118 |
| **Islet-1** | GCCTCAGTCCCAGAGTCATC | AGAGCCTGGTCCTCCTTCTG | 308 |
| **Mlc-2v** | GACCCAGATCCAGGAGTTCA | AATTGGACCTGGAGCCTCTT | 163 |
| **Nanog** | CACCCACCCATGCTAGTCTT | ACCCTCAAACTCCTGGTCCT | 152 |
| **Nkx2.5** | CAAGTGCTCTCCTGCTTTCC | GTCCAGCTCCACTGCCTTCT | 130 |
| **NPR-A** | CCAATTATGGCTCCCTGCTA | CGGTACAAGCTCCCACAAAT | 198 |
| **NPR-B** | TCATGACAGCCCATGGGAAA | GGTGACAATGCAGATGTTGG | 209 |
| **Oct-4** | GGATGCTGTGAGCCAAGG | GAACAAAATGATGAGTGACAGACAG | 175 |
| **PDGFRα** | GGGAAGGACTGGAAGCTTGGGGC | AGATGAGGCCCGGCCCTGTGAGG | 154 |
| **Sca-1** | TTTGAGACTTCTTGCCCATC | ACCCAGGATCTCCATACTTTC | 159 |
| **Sox2** | AAGGGTTCTTGCTGGGTTTT | AGACCACGAAAACGGTCTTG | 150 |
| **Tbx5** | GGAAAGATGAGGAATGTTCCAG | GTGTTACAGCTGATGTCCTCCA | 223 |
| **Tbx20** | CCCCGCTGCCAGCCAGGCTCTA | GTGCACCCGTGGCTGGTACTTATGC | 167 |
| **Tcf21** | GGCCAACGACAAGTACGAGA | GCTGTAGTTCCACACAAGCG | 129 |
| **Troponin T** | GCGGAAGAGTGGGAAGAGACA | CCACAGCTCCTTGGCCTTCT | 127 |
| **Vimentin** | GCCGAAAGCACCCTGCAGTCA | GCCTGCAGCTCCTGGATCTCTTCA | 146 |
| **vWF** | GATGCCCCAGTCAGCTCTAC | TCAGCCTCGGACAACATAGA | 131 |
| **Wnt1** | CACGGCACAGGGTATGAGAG | GTTGGGGCCACTCCAGATAC | 128 |

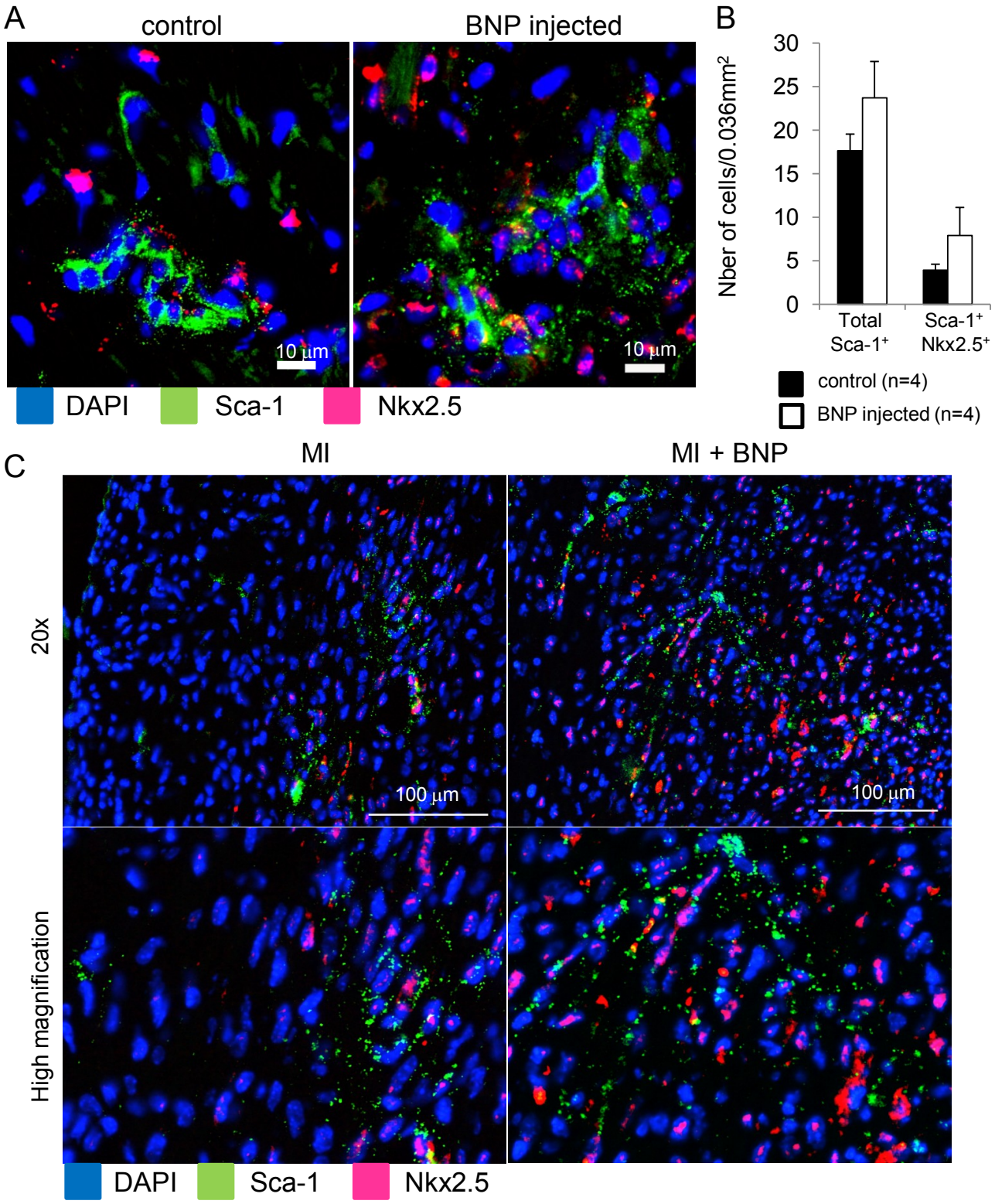
| | | | |
|---|---|---|---|
| **α-MHC** | AACCAGAGTTTGAGTGACAGAATG | ACTCCGTGCGGATGTCAA | 130 |
| **β-MHC** | ATGAGACGGTGGTGGGTTT | CTTTCTTTGCCTTGCCTTTG | 117 |
| **18S** | ACTTTTGGGGCCTTCGTGTC | GCCCAGAGACTCATTTCTTCTTG | 96 |



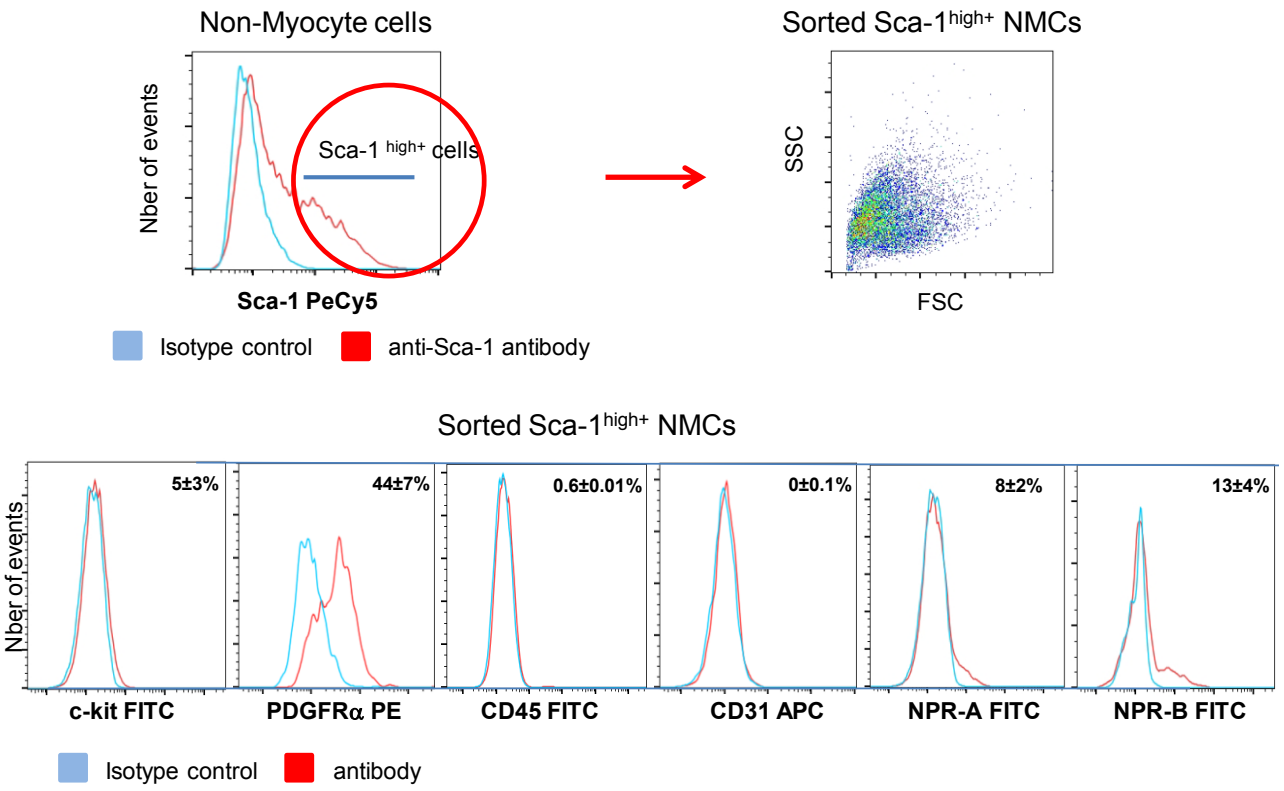

**Supplemental Figure 1. BNP induces Sca-1⁺ cell proliferation. A.** Quantitative relative expression of mRNA coding for Sca-1 in untreated and BNP treated cells. n= at least 7 different experiments per group. **B.** Representative immunostaining of cells cultured 4 days with BNP and stained with antibodies against the Proliferating Cell Nuclear Antigen (PCNA) (pink), Sca-1 (green) and DAPI (blue). White arrows highlight proliferating Sca-1⁺ cells. Scale bar represents 100 μm.



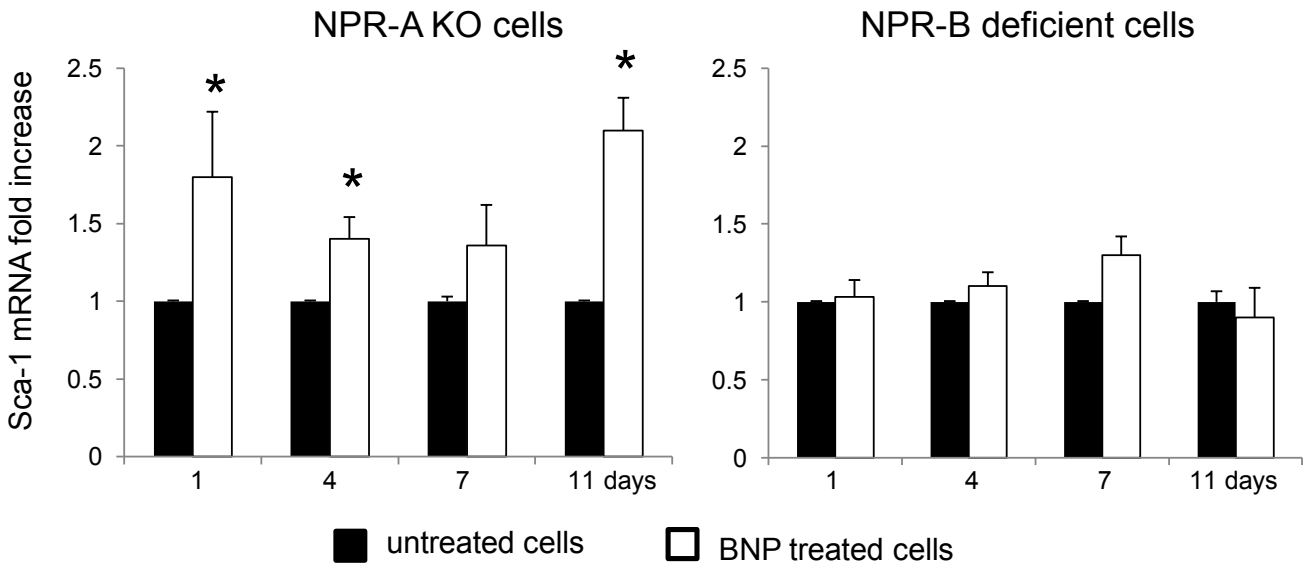

**<u>Supplemental Figure 2.</u> NPR-A and NPR-B receptors are expressed on Nkx2.5$^{+}$ Sca-1$^{+}$ cells in neonatal and adult hearts.** Immunostainings of neonatal (A) and adult murine hearts (B). Scale bars represented 10 μm. The yellow arrows point to Sca-1$^{+}$ Nkx2.5$^{-}$ cells expressing NPR-A or NPR-B, the white arrows point to Nkx2.5$^{+}$ Sca-1$^{+}$ cells and the red arrows point to Sca1$^{-}$ cells expressing Nkx2.5 and NPR-B.



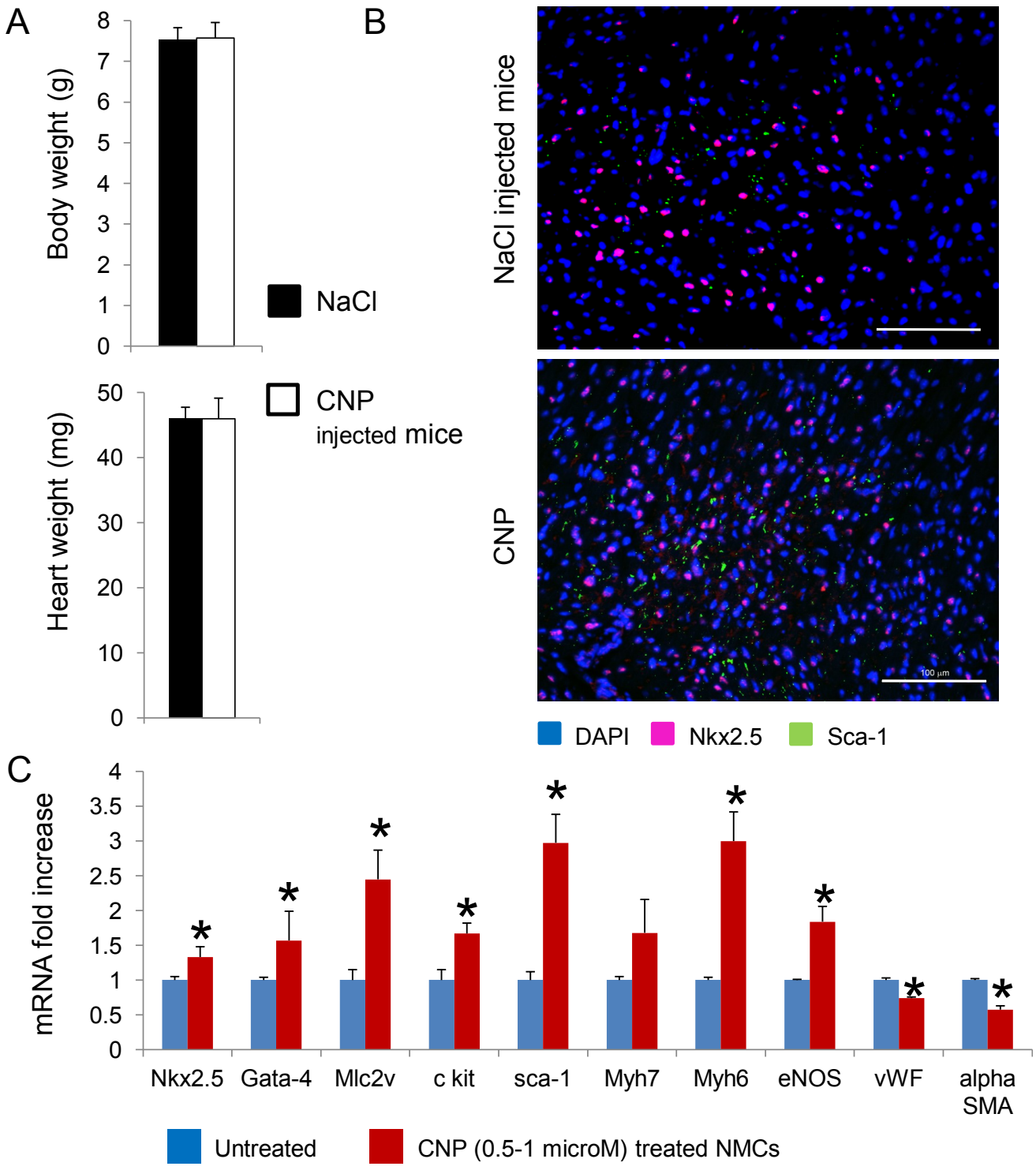

**<u>Supplemental Figure 3.</u> BNP treatment in neonatal and infarcted hearts leads to increased number of Sca-1⁺ Nkx2.5⁺ cells**. **A.** Heart sections from control or BNP injected neonatal mice. **B.** Quantification of the number of Sca-1⁺ cells expressing Nkx2.5 in the hearts of control or BNP injected mice. Data were obtained from at least 6 different pictures per mouse, *n* = 4 mice per group. Each section covered an area of 0.036 mm². Data are means ± SEM. **C.** Heart sections, from infarcted mice injected or not with BNP. Representative pictures of x 20 and high magnification of the infarcted area of the infarcted hearts 10 days afer myocardial infarction (MI).



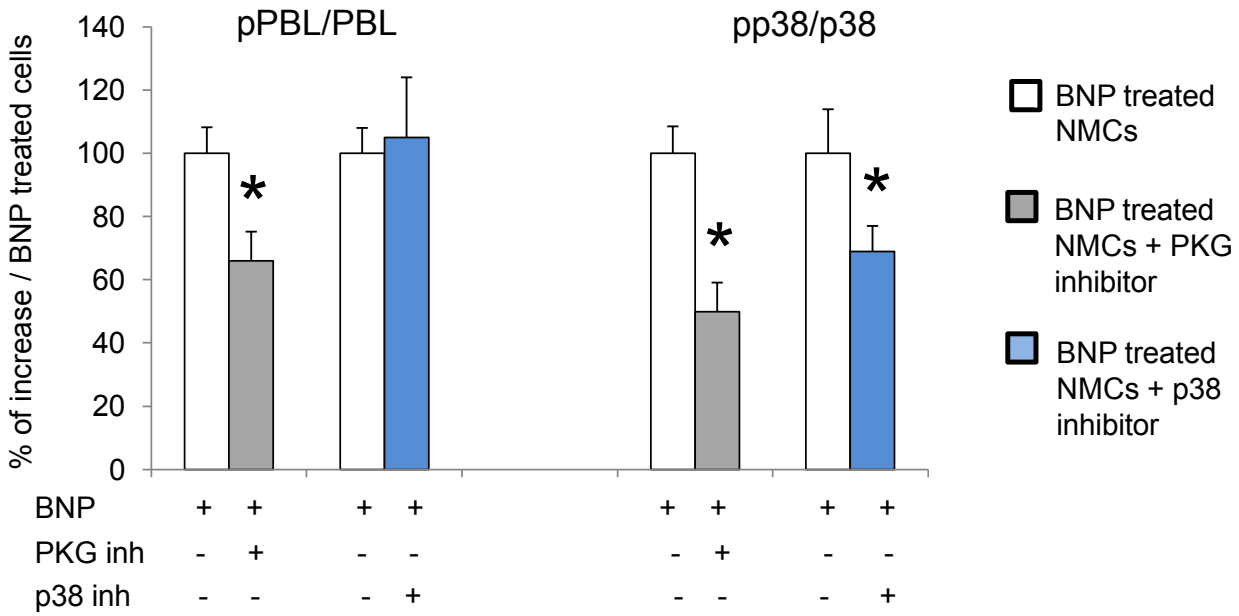

**<u>Supplemental Figure 4.</u> Phenotypic characterisation of the sorted Sca-1$^{high+}$ cells.** Representative histograms and dot plot (right) of non myocyte cell (NMC) sorting for high Sca-1 expression. The histograms allow to characterize the Sca-1$^{high+}$ cells for the expression of c-kit, PDGFRα, CD45, CD31 and NPR-A and NPR-B proteins. The numbers represent the percentage of the positive cells compared to the total number of Sca-1$^{high+}$cells and are the means ± SEM of 3-11 different experiments.



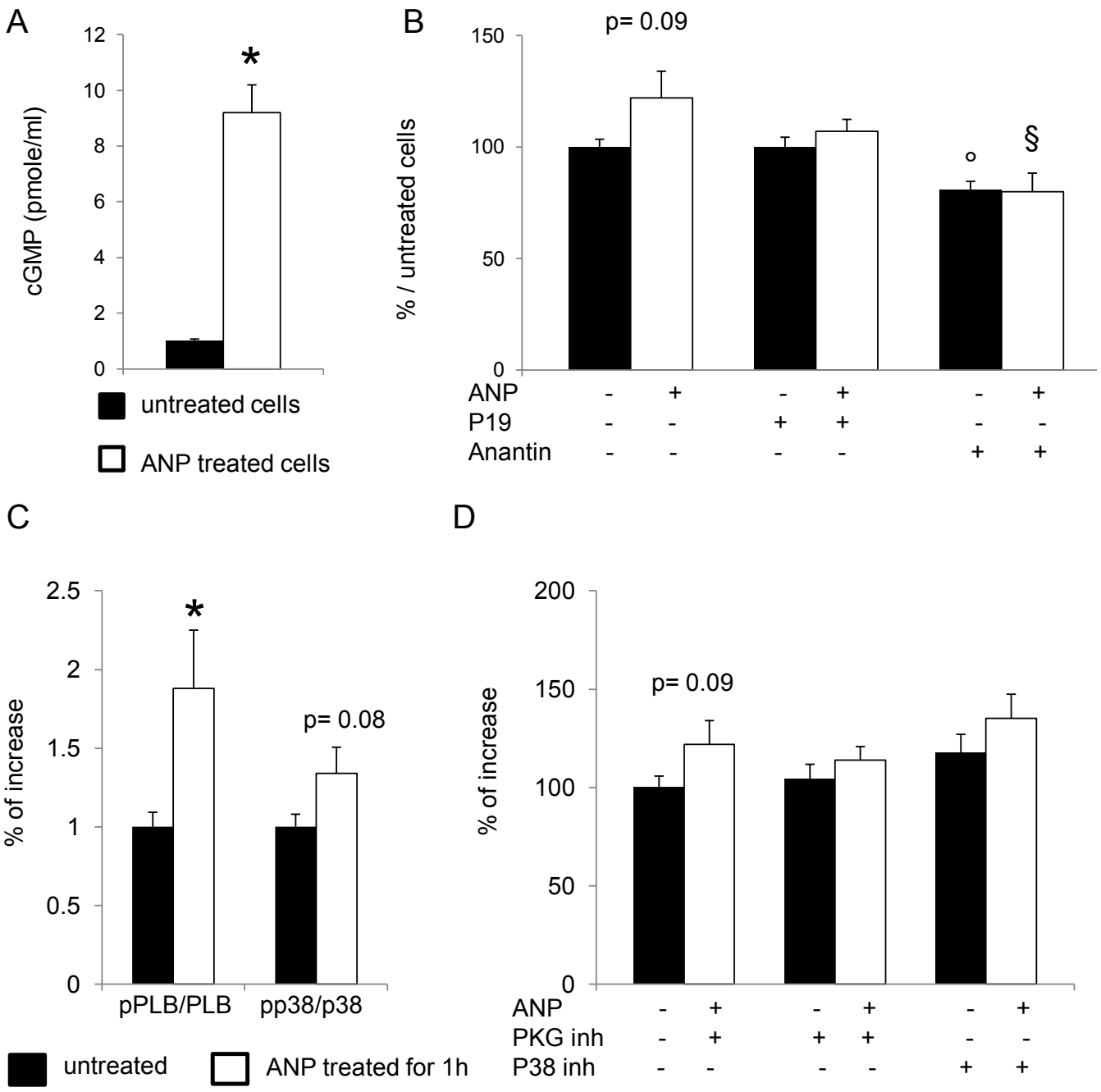

**Supplemental Figure 5. BNP induced increased Sca-1 mRNA expression only in presence of NPR-B receptor.** Quantitative relative expression of mRNA coding for Sca-1 in untreated and BNP treated NPR-A and NPR-B deficient NMCs (n= at least 6 different experiments at different time point). All the results are means ± SEM, * $p<0.05$ versus untreated cells.




**Supplementary Figure 6. CNP effect on cardiac Non Myocyte Cells *in vivo* and *in vitro*.**
**A-B.** Newborn mice (4 days old) were injected with CNP intraperitoneally every 2 days for up to 2 weeks. Mice were sacrificed 2 days after the last injection. CNP treatment had no effect on body or heart weights. However the numbers of Sca-1$^+$ cells and of Sca-1$^+$ Nkx2.5$^+$ cells was increased (**B**). n=4 mice per group **C.** Non Myocyte cells were isolated from neonatal hearts and treated with CNP in differentiation medium for up to 3 weeks. In presence of CNP, NMCs upregulated cardiomyocyte specific genes (Nkx2.5, Gata-4, Mlc-2v, Myh6). n= 9 different experiments and
* p< 0.05. vWF: von Willbrand Factor, SMA : smooth muscle actin.




**Supplemental Figure 7. BNP induced PKG activation, which is necessary for at least a part of p38 MAPK phosphorylation.** NMCs were treated 15-30 min with BNP and with a PKG inhibitor or a p38 inhibitor. n= at least 6 different experiments per group. The data were related to the average of BNP treated NMCs. All the results are means ± SEM, * $p<0.05$ versus BNP treated cells.




**<u>Supplemental Figure 8.</u> ANP has a moderated effect on Sca-1$^{+}$ cell proliferation. A.** cGMP levels were measured in the non myocyte cell supernatants (n=4-7) 1h after ANP treatment (1$\mu$M). **B.** Sorted Sca-1$^{high+}$ cells isolated from wild type hearts were cultured with ANP in presence or not of NPR-B receptor antagonist (P19, 0.5-1 microM) and of an ANF antagonist (Anantin, 0.2 microM). The number of cells was counted after 9-11 days of culture and the results were related to untreated cells. **B**. Quantification of the data from western blot analysis expressed relatively to the average of untreated cells. Sorted Sca-1$^{high+}$ cells were or not treated 1h with ANP. **C**. Number of sorted Sca-1$^{high+}$ cells after 11 days of culture with or without ANP and PKG or p38 inhibitor. The data were related to the average of untreated cells. **A-C**: n= at least 4 different experiments, All the results are means ± SEM, * p<0.05 versus untreated cells, ° p< 0.05 versus the untreated cells without inhibitors, § p< 0.05 versus the ANP treated cells without inhibitors.