SUPPLEMENTAL MATERIAL

Experimental procedures

Human pulmonary artery smooth muscle cells (HPASMCs) were purchased from PromoCell (Heidelberg, Germany, cells from two individuals) and Invitrogen (Karlsruhe, Germany, cells from one individual). Staurosporine-treated HeLa cell lysate was purchased from Abcam (Cambridge, UK).

siRNA transfection

Human pulmonary artery smooth muscle cells (HPASMCs) were transiently transfected with nestin siRNA (sc-36032, Santa Cruz, Heidelberg, Germany) using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Karlsruhe, Germany). To transfect HPASMCs, cells were plated in a six well dish so they were approximately 80% confluent at the time of transfection. To transfect the cells, two sterile 1.5 ml tubes were prepared per sample. 100 µl of Opti-MEM® transfection medium (Gibco, Invitrogen, Karlsruhe, Germany) was pipetted into each of the tubes. To one of them 2 µl of Lipofectamine RNAiMAX transfection reagent were added and mixed gently. After 15 min incubation at room temperature, the nestin siRNA was added to the other medium-containing tube and mixed by repeat pipetting. The content of both tubes was were combined and incubated for 20 min at room temperature. The mixture was gently pipetted drop-wise onto the plated cells and the plate was gently rocked back-and-forth in order to evenly spread the siRNA. After 4h, the medium was changed. For every set of siRNA treated cells one sample with scrambled siRNA (Am4611, Ambion, Austin, TX) at the same concentration was prepared as a negative control.

Assessment of cell number

Cell number was assessed using two different methods complementing each other. After 24 hours in culture, the transfected cells were trypsinized and counted using a hemocytometer. 100,000 cells of each sample were plated on a new 6-well dish and 5000 cells per well of each sample, were plated on a 96-well cell culture dish in quadruplicates. After 72 hours, cells in the 96-well dish were subjected to XTT assay and those in the 6-well dish, were counted.

XTT assay

96 hours after transfection with nestin siRNA (i.e. 72 hours after re-plating the cells on a 96-well dish), cellular metabolic activity was assessed as a readout for cell number by an XTT assay as per the manufacturer's instructions (Cell Proliferation Kit II (XTT), Roche, Mannheim, Germany). Cells were seeded in quadruplicates.

Cell counting

Parallel to XTT assay, cells in the 6-well-dish were trypsinized again and counted with a hemocytometer. To check for changes of cell number of HPASMCs in the absence of nestin,

100,000 cells were plated in two sets of wells after transfection. From the 1st set of wells, cells were counted after overnight incubation (16h) to determine the number of viable cells while from the other set, cells were counted after 72 hours.

CLARITY

Lung tissue from nestin-GFP mice fixated in 4% PFA and embedded in Tissue-Tek (Sakura Finetek, Netherlands) was processed following protocols modified from Chung [1]and Yang[2]. Briefly, a manually cut tissue slice (> 300 μm) was incubated in Hydrogel Solution (acrylamide 4% in PBS, bisacrylamide 0.05% in PBS and 0.25% thermoinitiator V-50 (Sigma-Aldrich, Hamburg, Germany) for 24 h at +4°C. The hydrogel was allowed to polymerize at 56°C for 2 h and cleared in “Clearing Solution” (SDS 10%, boric acid 200mM, pH 7.4 in water) for 72 h at room temperature. After extensive rinsing with PBS-Triton 0.1 %, tissue was incubated with chicken anti-GFP (Novus, Abington, UK 1:100) for 5 d followed by 5 rinsing steps in 24 h in PBS-Triton. FITC-labeled donkey anti-chicken (Jackson, Suffolk, UK; 1:100) was used as secondary antibody and incubated for 5 d. After 5 rinsing steps (24 h in total), tissue was incubated at least 24 h in RIMS (refractory index matching solution: Histodenz 88% (D2158, Sigma) in 0.02M PB with 0.1% tween-20 and 0.01% sodium azide, pH 7.5) until it became transparent and embedded in RIMS. Imaging was performed on a Zeiss LSM 710 Confocal Laser Scanning Microscope and planes were captured at 4.8 μm distance.

References

1.. Chung K, Wallace J, Kim SY, Kalyanasundaram S, Andalman AS, Davidson TJ, Mirzabekov JJ, Zalocusky KA, Mattis J, Denisin AK, Pak S, Bernstein H, Ramakrishnan C, Grosenick L, Gradinaru V, Deisseroth K. Structural and molecular interrogation of intact biological systems. *Nature*. 2013 May 16;497(7449):332-7.

2. Yang B, Treweek JB, Kulkarni RP, Deverman BE, Chen CK, Lubeck E, Shah S, Cai L, Gradinaru V. Single-cell phenotyping within transparent intact tissue through whole-body clearing. *Cell*. 2014 Aug 14;158(4):945-58

Supplemental Figure Legends

Supplemental Video. Nestin-GFP localization in a 384 µm-thick slice of adult mouse lung. The video shows a sequence of pictures captured from a 384 µm slice of cleared lung tissue with the focus level shifting by 4.8 µm in each step. Nestin-GFP is exclusively expressed in the lung vasculature (red arrows) whereas the parallel running bronchioli showed no expression for nestin-GFP (white arrows).

Supplemental Figure S1. Analysis of SMA expression after hypoxic treatment in mouse lung. **A:** Western blot (60 µg cytosolic protein) show constant SMA expression during hypoxic exposure between 1d and 3w. Vinculin was used as loading control.

Supplemental Figure S2. Analysis of nestin expression and VSMC proliferation in postnatal development of mouse lung. A: Western blot (60 µg cytosolic protein) showing increased nestin and decreased calponin expression between d3 and d10 whereas SMA is mostly unchanged. β-Actin was used as loading control. B-C: Nestin-GFP expression comparing postnatal d1 and adult mice showed a higher nestin-GFP expression in d1 lung tissue. D-I: PCNA immunostaining. PCNA+ (proliferating) VSMCs (arrows) are clearly visible in lung samples from postnatal d1 (D), d3 (E), d7 (F), d10 (G) and d15 (H). Lung section from adult mouse showing no PCNA+ VSMCs (I). Proliferating epithelial cells (arrowheads) served as internal positive control. Arteries are marked by “a”.

Supplemental Figure S3. Nestin expression in pulmonary artery and aorta of rats after MCT treatment compared to controls. Western blot showing increased nestin expression in the pulmonary artery after MCT treatment (MCT) compared to untreated animals (control). In contrast, constant levels of nestin expression in the aorta of the MCT-treated (MCT) and untreated (control) animals were found (60 µg cytosolic protein). Vinculin was used as loading control.

Supplemental Figure S4. Isolated VSMCs. Nestin effects on cell number and nestin expression in isolated VSMCs (HPASMCs). **A:** Western blot showing nestin and the apoptosis marker cleaved PARP (89kDa) after treatment of nestin siRNA-treated and control (scrambled siRNA) cells. 80 µg total protein. Vehicle-and stauroporine-treated HeLa-cells served as control for apoptosis. Vinculin was used as loading control. **B:** XTT assay suggests a significantly lower cell number in case of nestin siRNA treatment. Bars show mean scores (± SEM) from 3 assessments. *P<0.0001. **C:** Cell counting: Cell number only increases in the presencence of nestin (scrambled siRNA) *P<0.01. There is no significant increase in cell number in the absence of nestin (nestin siRNA). Cells were counted after 16h (overnight) of siRNA treatment and after 72h of siRNA treatment. **D-I** (Immunostaining of untransfected HPASMCs): **D-F:** The proliferation marker Ki67 (red, arrow) is co-localized with nestin (green, arrowhead). (D: nestin, E: Ki67, F: merge). **G-I:** Different to nestin (green) calponin (red) is present only in a low number of cells displaying a more differentiated phenotype.

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