Supplemental Material

Transdifferentiation requires iNOS activation: Role of RING1A S-Nitrosylation

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Material and Methods

Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Other reagents included FBS, Taqman RT-PCR primers, Taqman master mix, DAF-FM diacetate, RNAimax and DAPI (Life Technologies, Carlsbad, CA); the TLR3 agonist polyinosinic:polycytidilic acid (PIC) and NFκB inhibitors Bay117082, celastrol and dexamethasone (Invivogen, San Diego, CA); antibody against the S-nitroso (SNO) moiety (Sigma-Aldrich); antibody against 3-nitrotyrosine (NT) (Cayman Chemical, Ann Arbor, Michigan); PE- antibody against human CD31 and APC- antibody against mouse CD144 (BD Biosciences, San Jose, CA); antibody against inducible nitric oxide synthase (iNOS), HRP conjugated goat anti mouse or rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); antibody against β-tubulin, Ring1A, H3K27 trimethylation (H3K27me3) and H3K9 dimethylation (H3K9me2; Abcam, Cambridge, UK); bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ); Scramble and iNOS siRNA (Integrated DNA Technologies, Coralville, IA).

Cell culture and iEC generation

BJ human neonatal foreskin fibroblast cells (BJ fibroblasts) (Stemgent), WT and iNOS-/- murine embryonic fibroblasts (MEF; Cell Biologics Inc, Chicago IL) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin (5% CO_2, 37°C). We transdifferentiated human and mouse fibroblasts to endothelial cells (iEC) using our previously established protocol [1]. Briefly, cells were treated with PIC (30 ng/mL) daily for 7 days, and the medium was gradually transitioned to a 10% knockout serum replacement. Cells were treated with endothelial cell growth medium 2 (EGM-2; Lonza) containing 20 ng/mL BMP4, 50 ng/mL VEGF, and 20 ng/mL bFGF for another 7 days. For maintenance of transdifferentiation, the cells were cultured for another 2 weeks in the presence of bFGF, VEGF, and 0.1 mmol/L 8-bromoadenosine-3':5'-cyclic monophosphate sodium salt (8-Br-cAMP), with the medium changed every 2 days. In some experiments, inhibitors of iNOS or NFkB were present during the first three days of the transdifferentiation protocol; they were re-added when the transdifferentiation medium was refreshed.

RNA extraction and Real-Time PCR

mRNAs were extracted from cultured cells using RNeasy Mini Kit (Qiagen). The mRNA was reverse transcribed into cDNA and then specific primers for targeted genes were used for PCR amplification. For real-time PCR, a SYBR-green PCR system or Taqman system was used and ran on QuantStudio real-time PCR system (Life Technologies). Gene expression data using the ΔΔCt method was normalized to the expression of the 18S gene and plotted as relative fold changes.

Western blot and Immunoprecipitation

Western blot analysis was performed as previously described [2]. Cultured cells were homogenized in RIPA buffer containing 1× protease and phosphatase inhibitor cocktail. Protein concentration was quantified using BCA assay (Pierce) and resolved on 4-12% gradient SDS-polyacrylamide gels. The gel was transferred into nitrocellulose membrane using an iBlot System (Life Technology). Blots were stained with 1% Ponceau S for loading controls. Then blots were blocked with 5% nonfat milk in PBST (PBS with 0.1% Tween 20) for 1 hr at room temperature and probed with primary antibody overnight or longer at 4°C. Blots were then washed 4 times with PBST for 10 min. Horse radish peroxidase (HRP) -conjugated goat anti-mouse or rabbit antibodies were incubated for 1 hr at room temperature. Blots were washed for 4 times with PBST for 10 mins. Then antigen-antibody complexes were detected by exposure in a Fluorchem M system (Proteinsimple, San Jose, CA).

Immunoprecipitation was performed using the Pierce Classic Magnetic IP/Co-IP Kit (Thermo Scientific, Waltham, MA) following manufacturer's manual. After exposure to a variety of experimental conditions, BJ fibroblasts were washed and collected in lysis buffer (pH 7.4, 0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP40, 5% glycerol). After 30 min incubation on ice, the lysate was centrifuged at 13,000×g for 10min to collect the supernatant. The cytosolic extract (500μg) was pretreated with protein A/G magnetic beads and incubated with control IgG, anti-SNO or anti-RING1A antibody overnight at 4°C. Protein A/G magnetic beads were incubated with samples for 2 h at 4°C, pelleted and washed three times with lysis buffer. Proteins were eluted in pH2.0 elution buffer and neutralized with pH8.5 buffer for western blot analysis.

Chromatin Immunoprecipitation (ChIP) -qPCR

ChIP was performed using a previously established method [1, 2]. Recovery of genomic DNA was calculated as the ratio of copy numbers in the immunoprecipitate to the input control. Primers of the CD144

promoter were custom designed and synthesized from IDT, as follows: primer sequence (5’ to 3’), (F): CAGCCTCTTGGTTCTTCTGG; (R): GGAGTCAAGTGACCCAGCTC.

Fluorescent assay for nitric oxide (NO)

To assess intracellular NO, we stained BJ fibroblasts using 4-Amino-5-methylamino-2’, 7’-difluorofluorescein Diacetate (DAF-FM DA). After the fibroblasts had been exposed to experimental conditions, they were incubated with DAF-FM DA 10 μM at 37 °C for 30 min. Fluorescence was quantified by measuring fluorescence intensity at 495 nm excitation and 515 nm emission by a fluorescence plate reader (Tecan M1000 PRO).

Immunofluorescent staining for iNOS and RING1A

After BJ fibroblasts were cultured in 8 well chamber slides and exposed to experimental conditions, cells were then fixed with 4% paraformaldehyde, permeabilized with phosphate-buffered saline (PBS) with 0.1% Triton X-100, blocked with 5% normal donkey serum, and stained for anti-human RING1A or iNOS antibody overnight at 4°C. After washes with PBS, the cells were further incubated with Alexa Fluor 488 or 555 conjugated secondary antibodies at RT for 1 hr. The nuclei were stained with DAPI for 10 min.

Analysis of S-nitrosylation by mass spectrometry (MS)

Protein from both control and PIC treated BJ fibroblasts was immunoprecipated using RING1A antibody, resolved on 4-12% gradient SDS/PAGE gel and stained with Coomassie Blue. After destaining, the RING1A band was cut and sent to the mass spectrometry-proteomics core facility (Baylor College of Medicine). The gel band was further destained and double digested with trypsin and GluC (NEB) in situ. Peptides in the gel were extracted, quantified and 200 ng of each sample was analyzed using the Eksigent nanoLC system and then the ABCIEX TripleTOF 5600 mass spectrometer. The Ekisgent nanoLC and the ABCIEX TripleTOF 5600 mass spectrometer were controlled by Analyst software version 1.6 (ABCIEX Inc.). The MS spectra (m/z 100-2000) were acquired with internal mass calibration. Raw data were converted to MGF format and then imported into Peaks software v6.0 for further peak generation and database search with the SPIDER function. The search sequence was E3 ubiquitin-protein ligase RING1A of human (NP_002922.2 GI:51479192).

Construction of RING1A overexpression plasmid and site directed mutagenesis

Human RING1A cDNA fragment was amplified with forward primer (5'-CAGGAATTCGAATGACGACGCCGGCGAATG-3') and reverse primer (5'-GAGGTCGACGTCACTTTGGATCCTTGGTGGGAGC-3') from pCMV6-XL4-RING1A (OriGene, # SC118280) and ligated into EcoRI/SalI digested pCMV-Tag2C to generate pCMV-Tag2C-RING1A. We further created pCMV-Tag2C-RING1A-C398A using a PCR-based method [3, 4]. Briefly, PCR reaction was set by 10 ng of template (pCMV-Tag2C-RING1A), 1 μM primer pair: C398A Mutation- Forward (5'-CGGCCACTGGAGCTGGCCTATGCTCCCACCAAGGATCCAAAG-3') and C398A Mutation-Reverse (5'-TTGGTGGGAGCATAGGCCAGCTCCAGTGGCCGGGACAC-3'), 200 μM dNTPs and 1 unit of Phusion High-Fidelity DNA Polymerase (NEB, # M0530S). The PCR cycles were initiated at 94°C for 5 minutes, followed by 18 amplification cycles. Each amplification cycle consisted of 94°C for 30 seconds, 68°C for 3 minutes. The PCR products were treated with 5 units of DpnI at 37°C for 2 hours and then 5 μl of each PCR reactions was transformed respectively into E. coli Top10 competent cells by heat shock. Single clones were sequenced to examine the C398A mutation.

Flow cytometry

Cells were trypsinized and filtered through a 40 μm nylon mesh to generate single cell suspensions. Cells were stained with antibody for cell surface marker detection. Data were analyzed by flowjo software.

Data analysis

Results are expressed as the mean±SEM. Statistical comparisons between 2 groups were performed via Student *t* test. One-way ANOVA was used to compare the means of multiple groups. $P<0.05$ was considered statistically significant.

References

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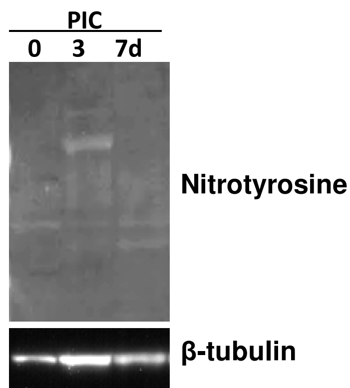
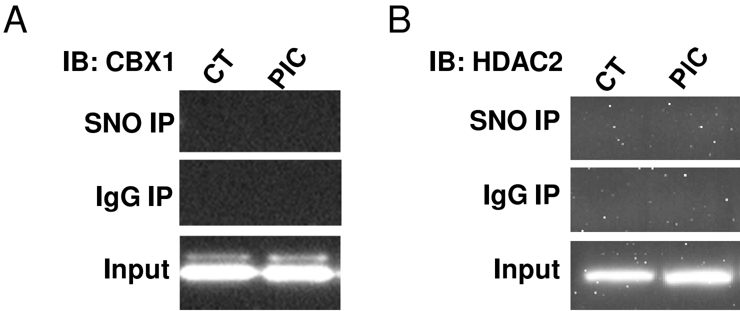
Supplemental Figures

Online Figure I



Online Figure I. 3-NT protein increased during transdifferentiation. BJ fibroblasts were treated with PIC for 0, 3 and 7 days and collected for western blot. Lysates from treated cells were subjected to immunoblotting with 3-NT antibody and β-tubulin antibodies.

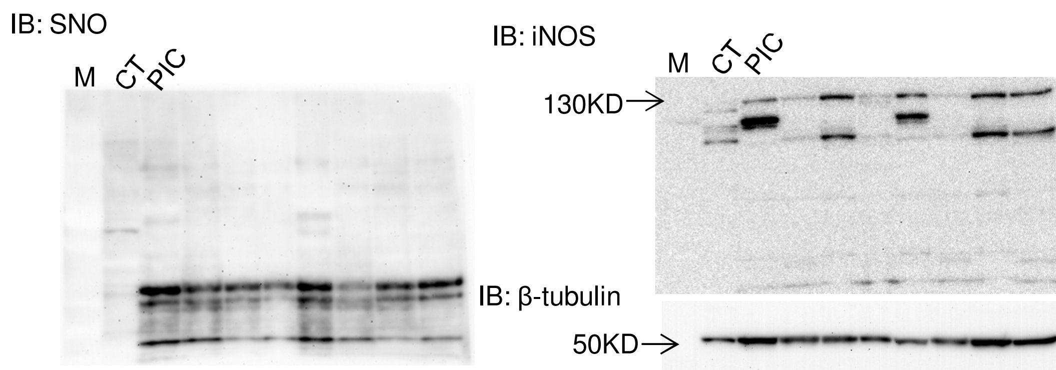
Online Figure II



Online Figure II. CBX1 and HDAC2 are not S-nitrosylated during transdifferentiation. BJ fibroblasts were treated with PIC or vehicle control for 3 days. Cell lysates were subject to immunoprecipitation with SNO antibody and then immunoblotted for (A) CBX1 antibody or (B) HDAC2 antibody.

Online Figure III

A



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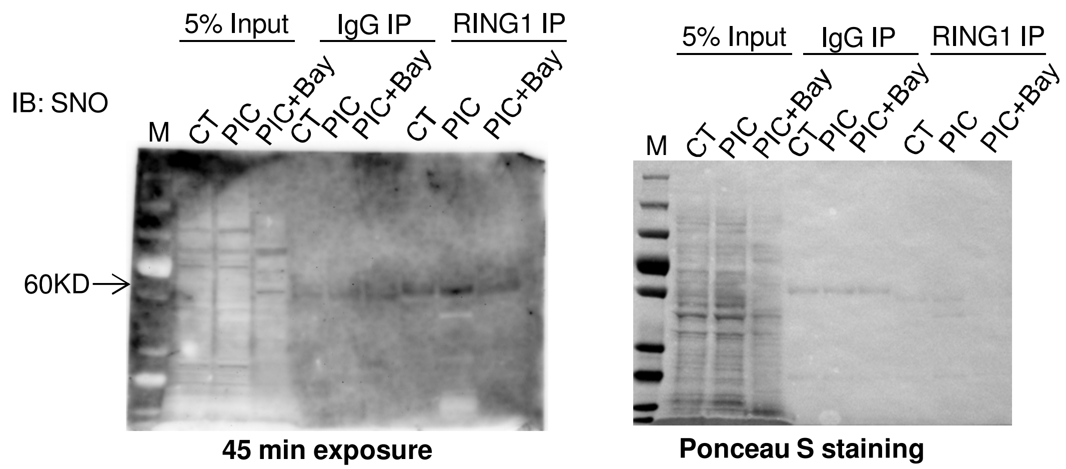
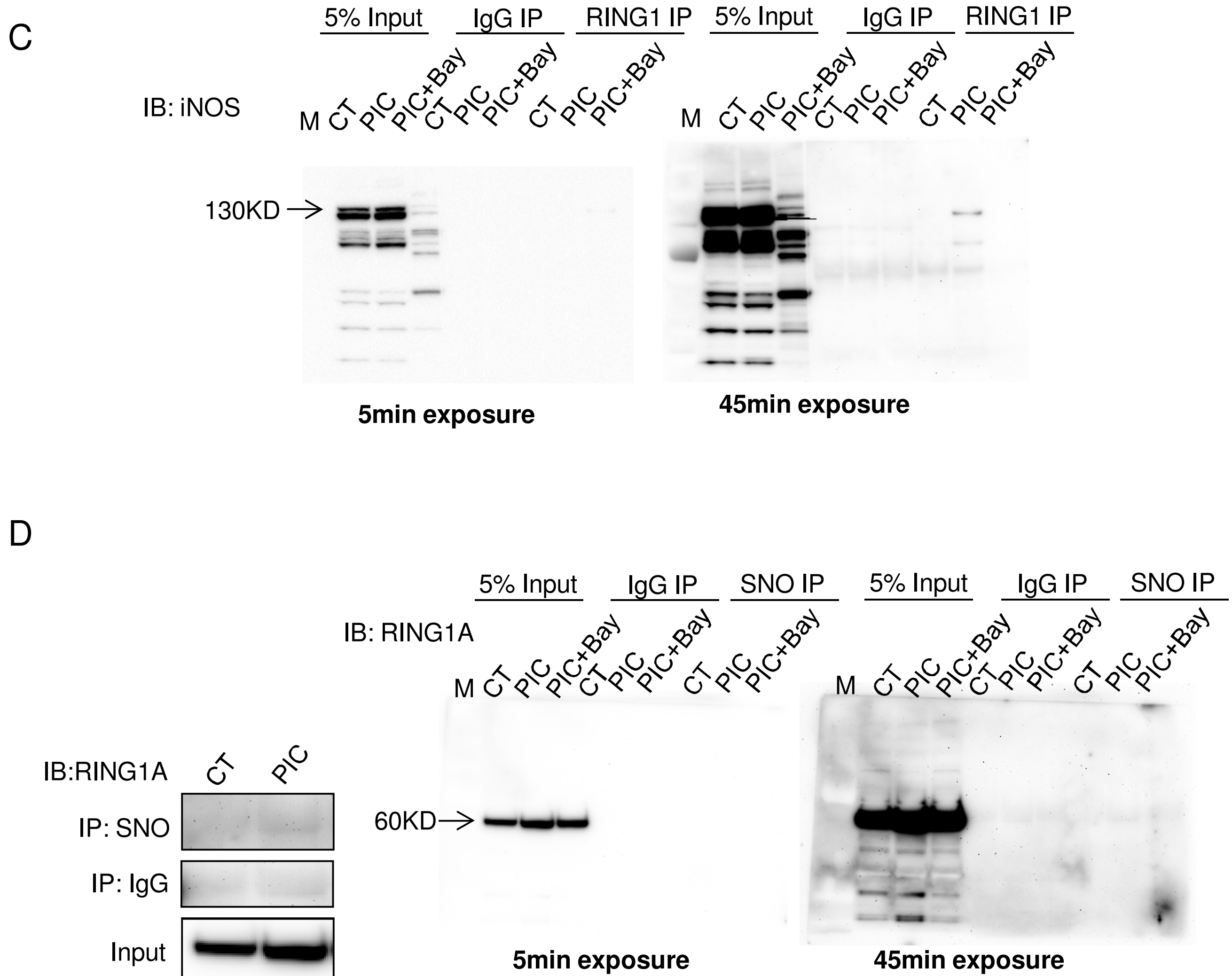
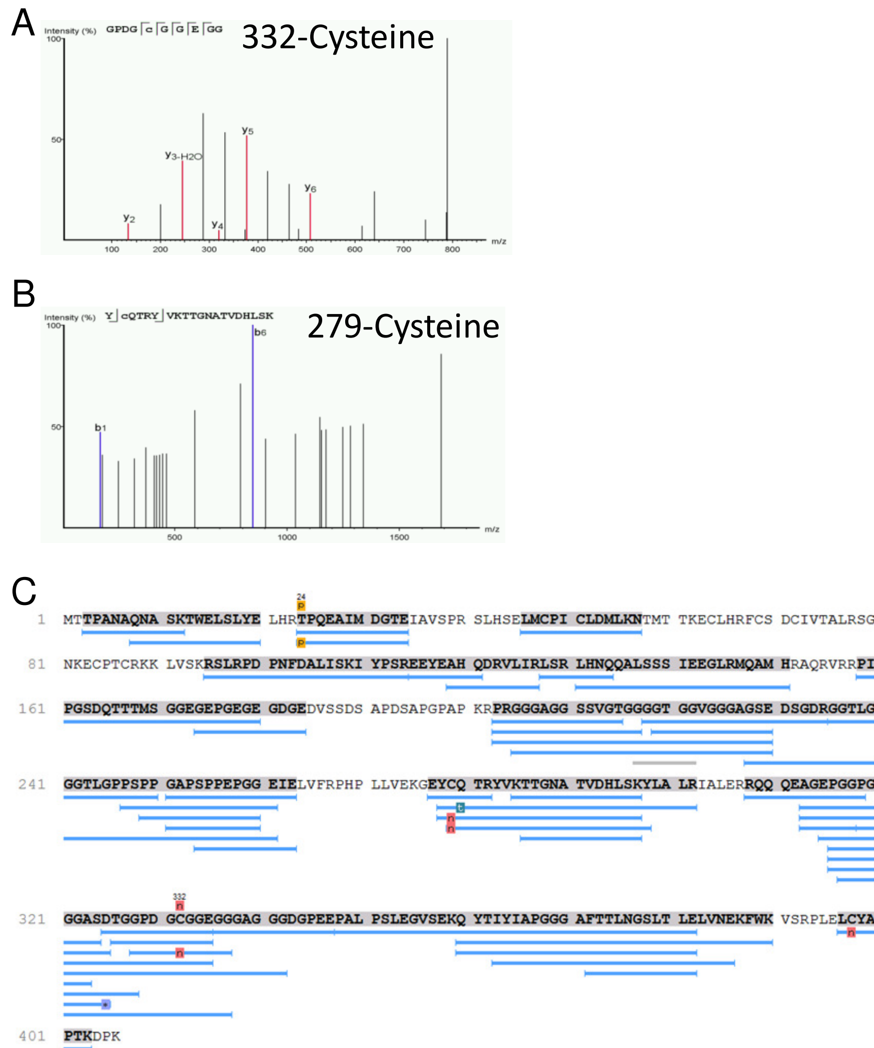


Online Figure III



Online Figure III. iNOS binds and S-nitrosylates Ring1A. BJ fibroblasts were treated with vehicle; PIC; or with PIC and the NFκB inhibitor Bay117082 for 3 days and proteins were collected for western blot or immunoprecipitation application. (A) Original blots of Figure 5A. (B) Original blots of Figure 5B. (C) Original blots of Figure 5C. (D) Proteins from cell lysate were immunoprecipitated with SNO or control IgG antibody and subject to western blot detection of RING1A. Left panel showed the western blot. Middle and right panels are the original blots.

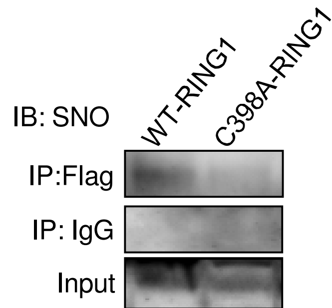
Online Figure IV



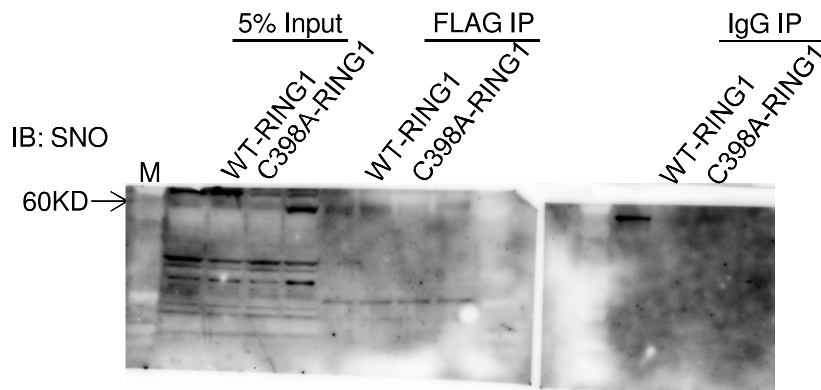
Online Figure IV. MS analysis of S-nitrosylation site of Ring1A. MS/MS fragmentation spectra for (A) the Cys332-containing peptide and (B) the Cys279-containing peptide. MS spectra of the cysteine-containing peptides identified after MS analysis. Peptide sequence is shown at the top left of each spectrum, with the annotation of the identified matched amino terminus-containing ions (b ions) in black and the carboxyl terminus-containing ions (y ions) in red. For clarity, only major identified peaks are labeled.

Online Figure V

A

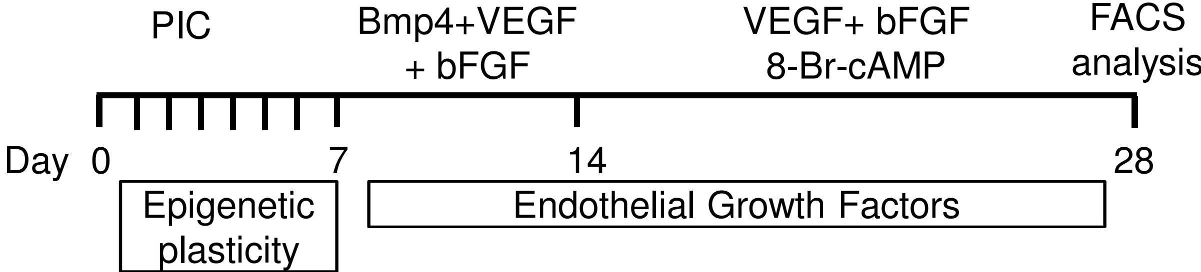


B



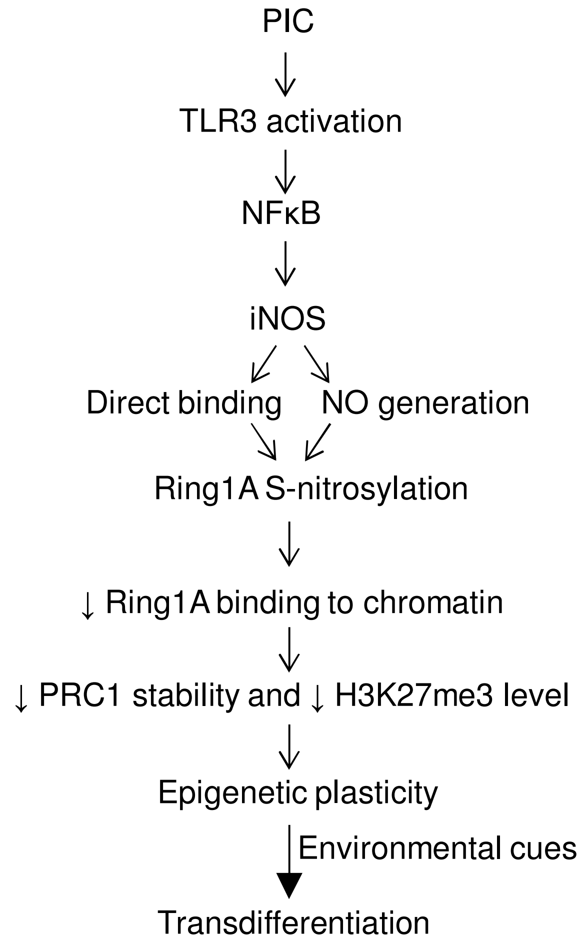
Online Figure V. RING1A-C398A mutation ablished S-nitrosylation. BJ fibroblasts were transfected with either pCMV-Tag2C-RING1A-C398A (WT-RING1A) or pCMV-Tag2C-RING1A-C398A (C398A-RING1A) plasmid and then subject to transdifferentiation protocol. At day 3, proteins were collected and immunoprecipated with Flag or control IgG antibody. Products were examined by western blot detection of SNO protein. (A) Western blot. (B) Original blots of A.

Online Figure VI



Online Figure VI. Transdifferentiation protocol. Direct reprogramming of human fibroblasts to functional endothelial cells via activation of innate immunity and microenvironmental cues. The figure outlines the time course and sequential treatments.

Online Figure VII



Online Figure VII. Mechanism of epigenetic plasticity induced by innate immune activation.