Supplemental Materials

Cathepsin K Activity Controls Injury-Related Vascular Repair in Mice

Lina Hu, MD; Xian Wu Cheng, MD, PhD, FAHA; Haizhen Song, MD, PhD; Aiko Inoue, MS; Haiying Jiang, PhD; Xiang Li, MD; Guo-Ping, Shi, DSc; Eiji Kozawa, MD, PhD; Kenji Okumura, MD, PhD; Masafumi Kuzuya, MD, PhD

From the Department of Community Healthcare & Geriatrics (H.J., X.W.C., H.S., A.I., H.J., X.L., M.K.), Orthopaedic Surgery (E.O.), and Cardiology (K.O.), Nagoya University Graduate School of Medicine, Nagoya Japan; the Department of Cardiology (X.L., X.W.C.), Yanbian University Hospital; the Department of Physiology and Pathophysiology (H.J.), Yanbian University School of Medicine, Yanji, China; The Department of Dermatology (H.S.), No.3 People Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China.

Corresponding author: Xian Wu Cheng, MD, PhD, FAHA

Department of Geriatrics,

Nagoya University Graduate School of Medicine,

65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan.

Tel: +81-52-744-2364; Fax: +81-52-744-2371;

Email: xianwu@med.nagoya-u.ac.jp or chengxw0908@163.com

Mice

The male CatK$^{-/-1}$ and wild-type (WT, CatK$^{+/+}$) littermates used in this study were 8 weeks old and weighed between 21 and 25 g. Mice were provided with a standard diet (Oriental Yeast) and tap water ad libitum throughout the experimental period. All animal experiments were performed in accord with the guidelines on animal care of the Nagoya University Graduate School of Medicine.

Models of the carotid artery injuries and tissue collection

The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Dainippon Pharmaceutical, Osaka, Japan). For the single injury model, the right common carotid artery of 9-week-old mice was ligated just proximal to their bifurcations as described (Figure 1A upper panel).[2] For the combination injury, a polyethylene cuff (length 2 mm, inside dia. 0.580 mm, outside dia. 0.965 mm; Becton Dickinson, Lincoln Park, NY) was applied just proximal to the ligated site (Figure 1A down panel).[3] In the specific experiments, a specific Cat inhibitor E64*d* (10 mg/kg; Sigma-Aldrich, St. Louis, MO), dissolved in dimethyl sulfoxide (E64*d* group) or vehicle (control group) alone was injected into the abdomens of CatK$^{+/+}$ mice every other day from the combination surgery, from 3 days before the surgery to 14 days the surgery.

At the indicated timepoints after the injury, the mice were euthanized by an overdose of sodium pentobarbital. For the biological analysis, the mice were perfused with isotonic saline at physiological pressure, and then the carotid artery was isolated and kept in RNAlater solution (for the genes assay) or liquid nitrogen (for the proteins assay). For the morphometry, after being immersed in fixative for 16 h (4°C), the arteries were embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo) and stored at −20°C.

Morphometric and immunohistologic analysis

We prepared cross-cryosections (5 μm) at 2 mm proximal to the ligated site and stained them with hematoxylin and eosin (H&E). The perimeters of the lumen, the internal elastic lamina (IEL), and the external elastic lamina (EEL) were obtained by tracing the contours on digitized images. We calculated the medial area by subtracting the area defined by the IEL from the area defined by the EEL, and we determined the neointimal area by subtracting the lumen area from the area defined by the IEL.

For immunohistochemistry, corresponding sections on separate slides were treated with rabbit polyclonal antibodies against α-smooth muscle actin (ASMA; 1:100, NeoMarkers, Fremont, CA), rat monoclonal antibodies against macrophages (Mac 3;

1:40, BD Pharmingen, San Diego, CA), and CD31 (1:100, BD Pharmingen). The sections were reacted with an alkaline phosphatase (AP)-conjugated secondary antibody against rabbit, rat, or mouse IgG (1:200, all from Vector Laboratories, Burlingame, CA), respectively, for 2 h at 4°C, and were then visualized with an AP substrate kit (Vector Laboratories) in accord with the manufacturer's instructions. Levamisole (Vector Laboratories) was used as an inhibitor of endogenous AP. Double immunofluorescence was performed using rabbit polyclonal Ki67 Ab-3 antibody (NeoMarkers) and mouse monoclonal anti-ASMA antibody (1:100), and the sections were visualized using Alexa Fluor 594-conjugated anti-rabbit IgG/fluorescein-conjugated streptavidin (1:50; Vector Laboratories). In addition, picrosirius red (PSR) was performed for collagen staining. The slides were mounted in glycerol-based Vectashield medium (Vector Laboratories) containing the nucleus stain 4′,6-diamidino-2-phenylindole.

We analyzed images of sections stained for collagen, ASMA, Ki67, and Mac-3 with the use of BZ-II Analyzer, Exe 1.42 software (Keyence, Osaka, Japan). Five cross-sections of vessels in each aorta were quantified and averaged for each animal. The results are reported as the percentage of the intima area that contained lesions. We set a threshold to automatically compute the positively stained area for each antibody or histochemical stain and then computed the ratio (percent) of the positively stained area to the total cross-sectional vessel wall area or intimal plaque lesion area studied.[4]

Gelatin and in situ zymography

For gelatin zymography, 20 μg of artery protein extract was mixed with SDS sample buffer without reducing agent and loaded onto a 10% SDS-polyacrylamide gene containing 1 mg/mL gelatin as described.[4]

In situ zymography was performed using in situ Zymo-Film (Wako Chemicals, Osaka, Japan). In situ frozen sections (4 μm) were placed on the film and incubated for 30 h at 37°C in a moisture box following the manufacturer's instructions. The specimens were then stained with Amido black 10B (Sigma) for 15 min and destained for 10 min. The areas of gelatinolytic activity were visualized under a light microscope.

Western blot analysis

Protein was extracted using a RIPA lysis buffer and Western blotted against antibodies for phospho-phosphoinositide 3-kinase (PI3K), t-PI3K, phospho-p38 mitogen-activated protein kinase (p38MAPK), total p38MAPK, total mammalian target of rapamycin (mTOR), phospho-mTOR, total Akt, phospho-Akt (Cell Signaling Technology, Beverly, MA), MMP-9, MMP-12, CatS, CatK (Santa Cruz Biotechnology Inc.), CystC, CatB

(Upstate), MMP-2 (Daiichi Chemical Co.), MMP-13 (Fuji Chemical Co.), TIMP-1, TIMP-2, CatL, and β-actin (Sigma-Aldrich; loading control).

Quantitative real-time gene expression assay

RNA was harvested from tissue with an RNeasy Fibrous Tissue Mini-Kit (Qiagen, Valencia, CA). The mRNA was reverse-transcribed to cDNA with an RNA polymerase reaction chain (PCR) Core kit (Applied Biosystems, Foster City, CA). Quantitative gene expression was studied using the ABI 7300 Real-Time PCR System (Applied Biosystems). All experiments were performed in triplicate. The sequences of primers for targeted genes were as described.[4, 5] The transcription of targeted genes was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Preparation of explants and migration assay

We prepared the aortic explants as described.[2] Briefly, thoracic aortas of male mice were removed and opened out, and the endothelium was removed by gentle abrasion. Following cutting into 1×1-mm explants, the explants were individually plated with the lumen side down into collagen type 1-coated 24-well plates and cultured in 500 μL of Dulbecco's modified Eagle's medium (DMEM) containing 0.1% bovine serum albumin (BSA), transferrin, insulin, and platelet-derived growth factor BB (PDGFBB, 50 ng/mL, PeproTech, London, UK). At the indicated timepoints, we performed a quantitative analysis of SMC sprouts at the edge of the explants with Beta 4.0.3 of Scion Image software, and the SMC migratory ability is expressed as sprouted total cell numbers and areas (average of five explants for each animal).[2] To characterize the cell migration from explants, we fixed and stained cells for smooth muscle α-actin.

Cell culture

We obtained vascular SMCs from the media of mouse aortas of both genotypes by the tissue explant method[6] and cultured them in DMEM supplemented with 10% fetal bovine serum and antibiotics. The obtained cells retained SMC characteristics (purity > 90%).

Cell migration and invasion assays

The SMC invasion assay was performed on Transwell (Costar) 24-well plates tissue culture plates as described.[6] The cells that invaded the outer side of the membrane were stained and counted in six to eight randomly chosen fields of the duplicated chambers at a magnification of ×200 for each sample.

Cell proliferation assay

In vitro, SMC proliferation was assessed with the Cell Titer 96AQ Assay kit (Promega, Madison, WI).[2] Cells were plated on collagen-coated 96-well plates at 5000 cells in 100 μL of 0.3% BSA/DMEM per well and incubated in DMEM with the presence of platelet-derived growth factor-BB (PDGF-BB, 50 ng/mL) for 48 h. Then, 20 μL of a mixture of tetrazolium compound and phenazine methosulfate was added (for an MTS assay), and the absorbance was determined at 492 nm. Experiments were performed five separate times for each group in triplicate.

Statistical Analysis

Data are expressed as means ± SEM. Student's *t*-tests (for comparisons between two groups) or one-way ANOVA (for comparison of two or three groups) followed by Tukey post hoc tests were used for statistical analyses. SPSS software version 17.0 (SPSS Inc., Chicago, IL) was used. A value of $p < 0.05$ was considered significant.

References

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Figure S1

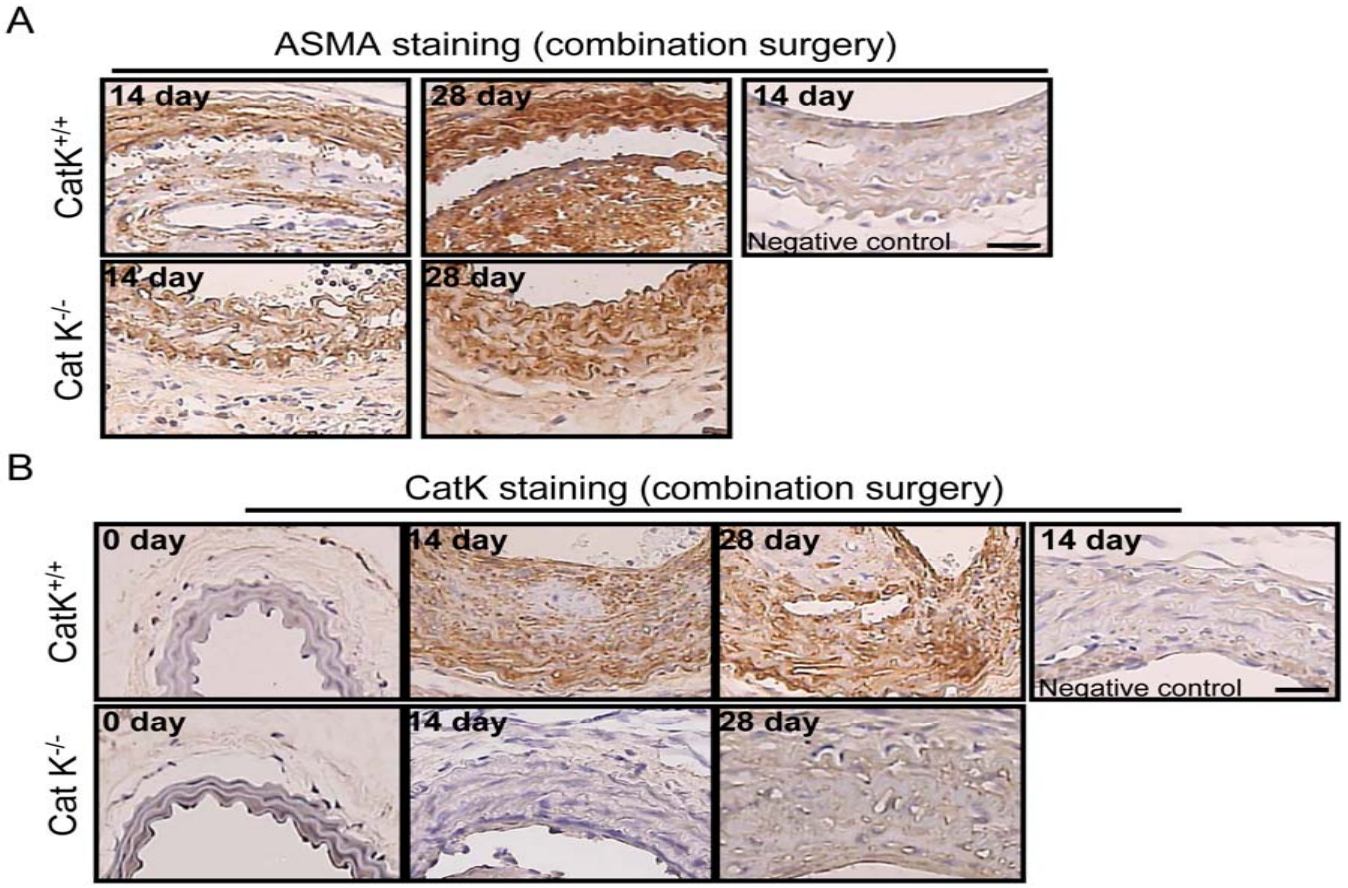

Figure S1. Representative immunostaining images of injured arteries from CatK+/+ and CatK−/− mice at days 0, 14, and 28 after combination surgery. A: Media and intimal lesion was positive for ASMA in CatK+/+ and CatK−/− mice at days 14 and 28 after combination surgery. B: CatK staining was positive for CatK in intimal and medial lesions. No staining was observed in adventitia in wild-type mice or in the whole arterial wall from CatK−/− mice. Negative control: staining without primary antibody. Bars = 100 μm.

Figure S2

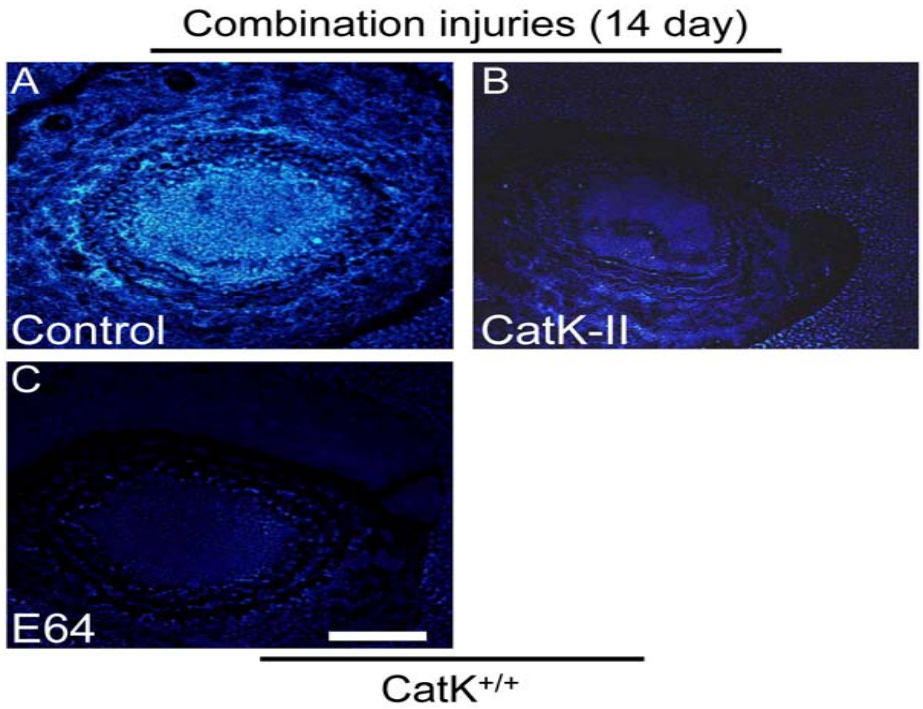

Figure S2. Gelatinolytic activity in the left carotid arteries of CatK+/+ mice. A–C: Representative images of in situ zymography at 14 days after combination injuries of CatK+/+ mice. Brightness under a light microscope shows degradation. For the Cat-dependent gelatinolytic activity assay, the gelatin films were incubated with or without the CatK specific inhibitor CatK-II (10 μM) and the nonspecific Cat inhibitor E64 (20 μM). Bars = 100 μm.

Figure S3

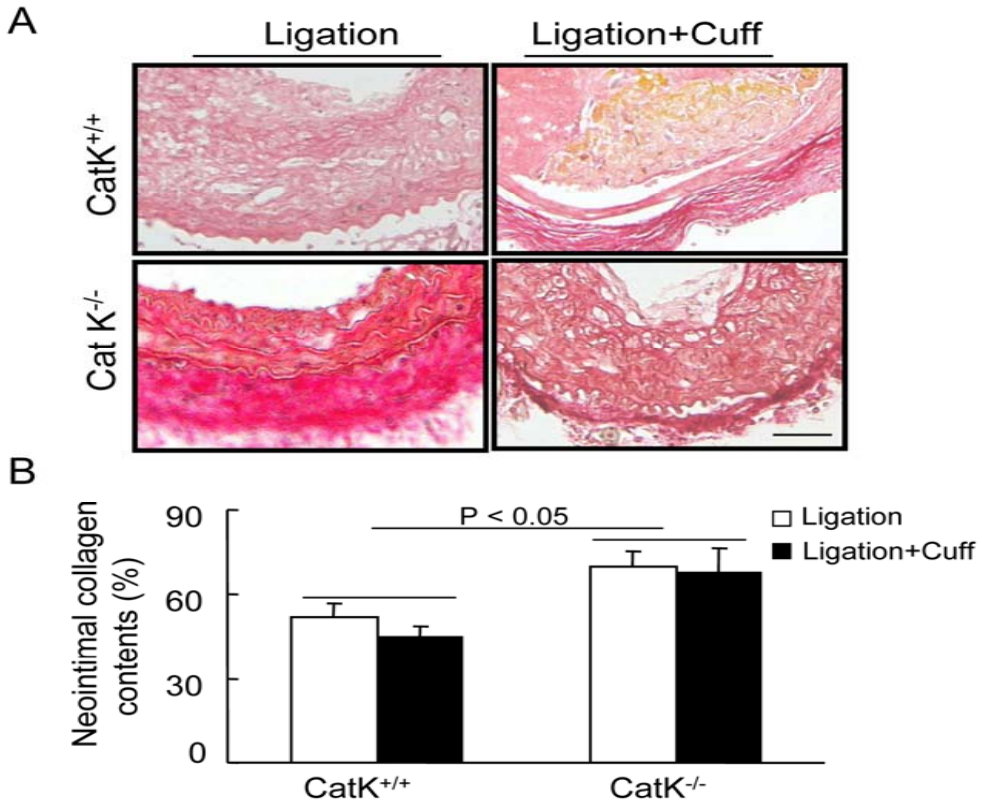

Figure S3. The contents of collagen in the injured arteries from both genotype mice. CatK deficiency enhanced collagen contents in either type of injury mice.

Figure S4

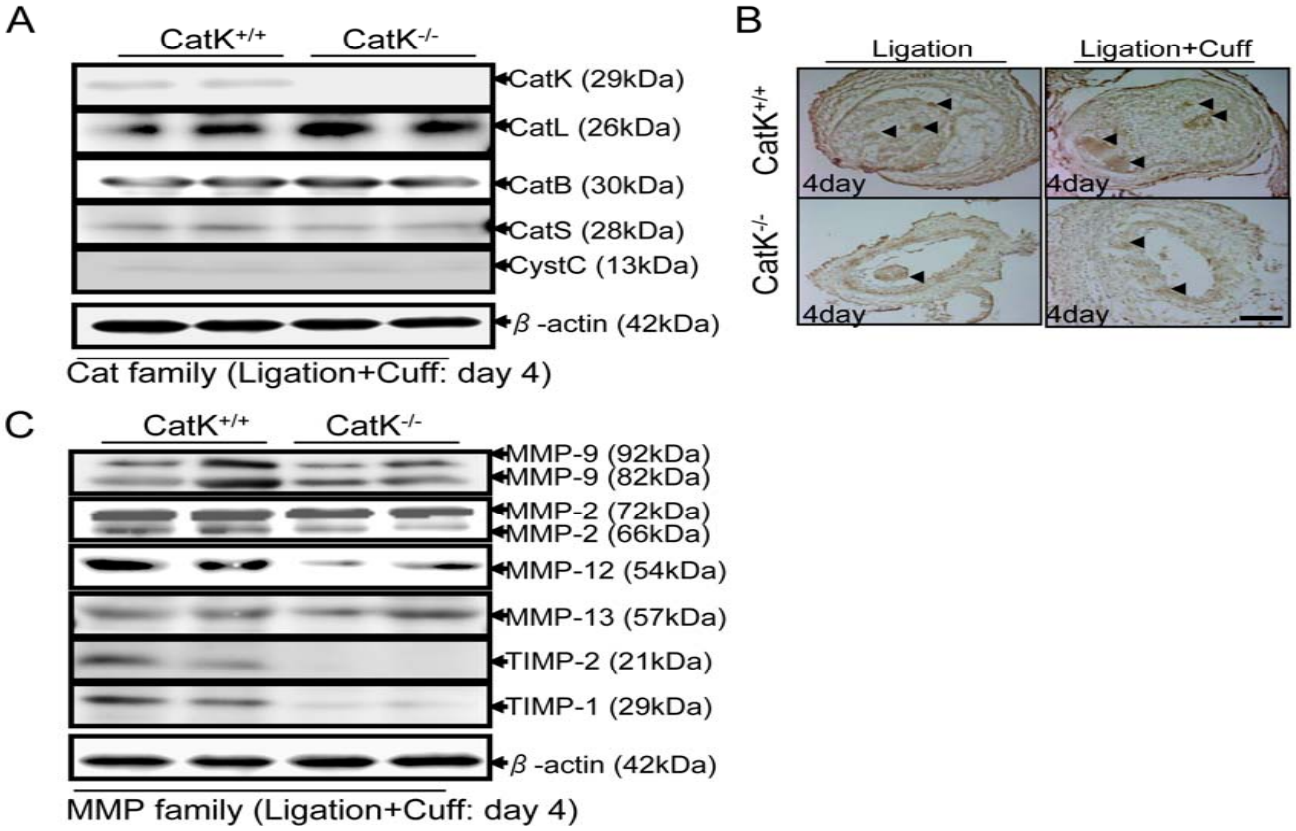

Figure S4. Targeted molecules protease family expression and macrophage infiltration in both genotype mice. A: Protein expressions of Cat family in combination injured arteries of $CatK^{+/+}$ and $CatK^{-/-}$ mice. B: Representative Mac-3 immunostaining image used to assess the content of infiltrated macrophages. C: Protein expressions of MMP family in combination injured arteries of $CatK^{+/+}$ and $CatK^{-/-}$ mice.

Figure S5

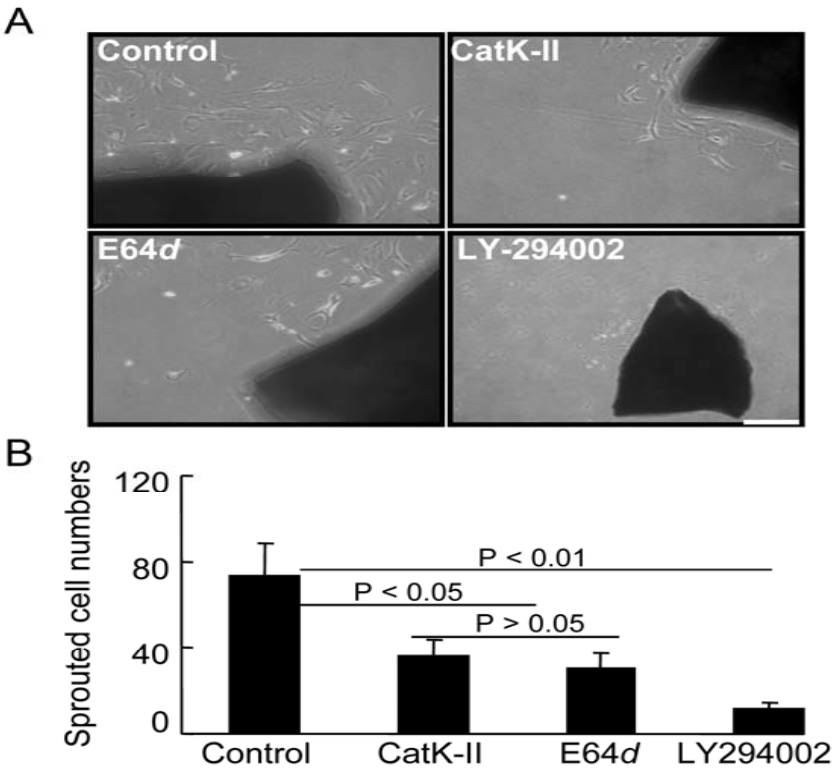

Figure S5. Cat inhibition impairs SMC migration *in ex vivo*. A-B: Representative images of SMC sprouting from arterial explants (A) and combined quantitative data for sprouted cell numbers (B) at day 7 are shown.

Figure S6

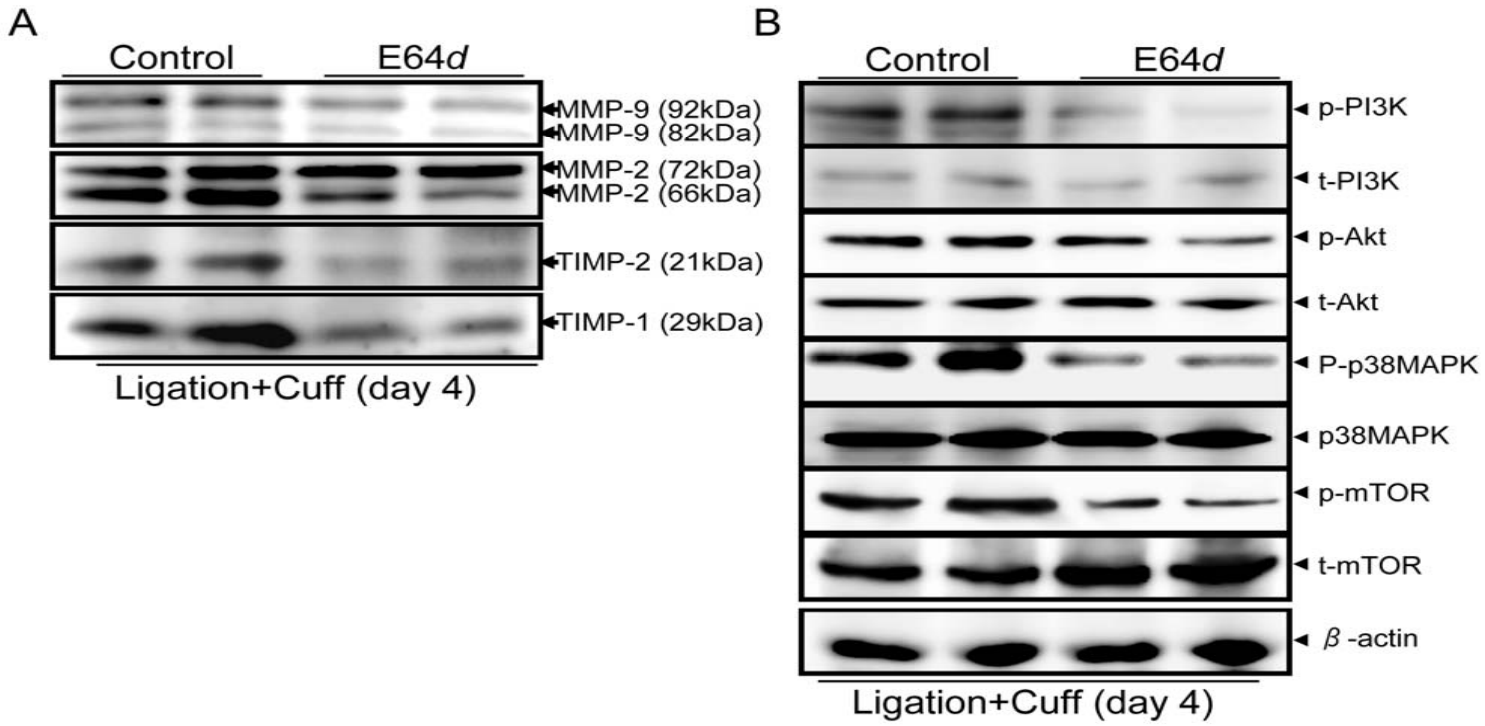

Figure S6. Effects of E64*d* on targeted signaling molecular phosphorylation and MMP family expression in double injured carotid arteries. A-B: The levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins as well as p-pI3K, p-Akt, p-p38MAPK, and p-mTOR proteins were lower in E64*d*-treated arteries than in that of untreated control arteries.

Figure S7

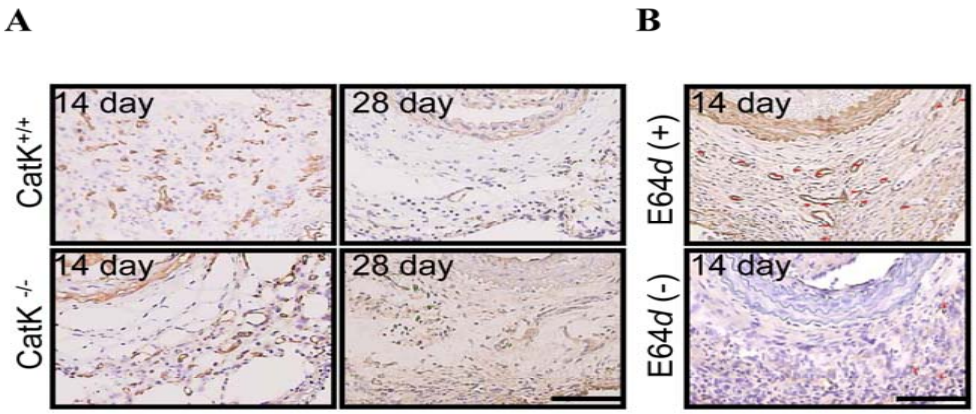

Figure S7. CatK$^{-/-}$ reduces neovessel density in neointima-related lesions. A and B: Representative CD31 immunostaining image used to assess the content of microvessels in injured arteries after double injuries.

Figure S8

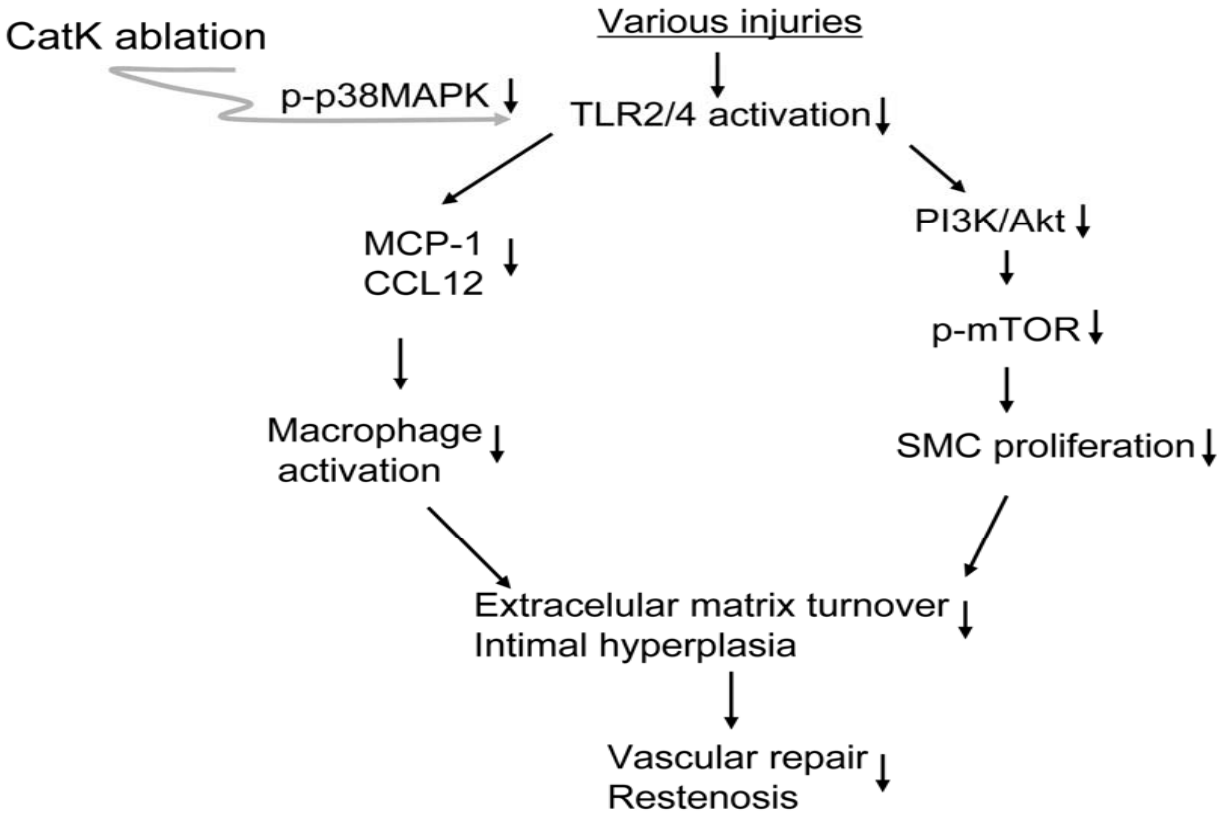

Figure S8. Proposed mechanism of the alleviation of vascular repair in the mouse carotid artery injury model. TLR2, toll-like receptor; MCP-1, monocyte chemoattractant protein-1, CCL12, chemokine (C-C motif) ligand 12; PI3K, phospho-phosphoinositide 3-kinase; p-p38MAPK, phospho-p38 mitogen-activated protein kinase; p-mTOR, phosphor-mammalian target of rapamycin; ? indicates unknown.