Supproting information

Nardilysin and ADAM proteases promote gastric cancer cell growth by activating intrinsic cytokine signaling via enhanced ectodomain shedding of TNF-α

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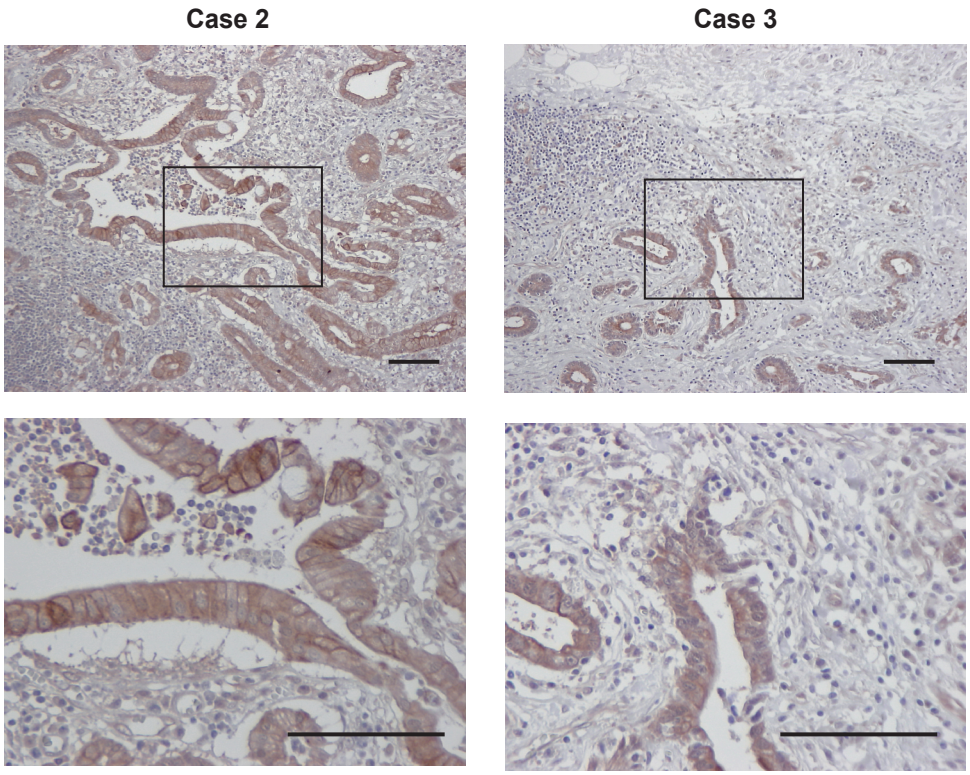

Figure S1
Immunohistochemical analysis of NRDc expression in gastric cancer tissues. Additional images of gastric cancer specimens immunostained with anti-NRDc antibody are shown. Lower panels are magnified photographs of the boxed areas in the upper panels. Left panels are images of another region of the case 2 specimen (See Fig 1B). Scale bars, 100 μm.

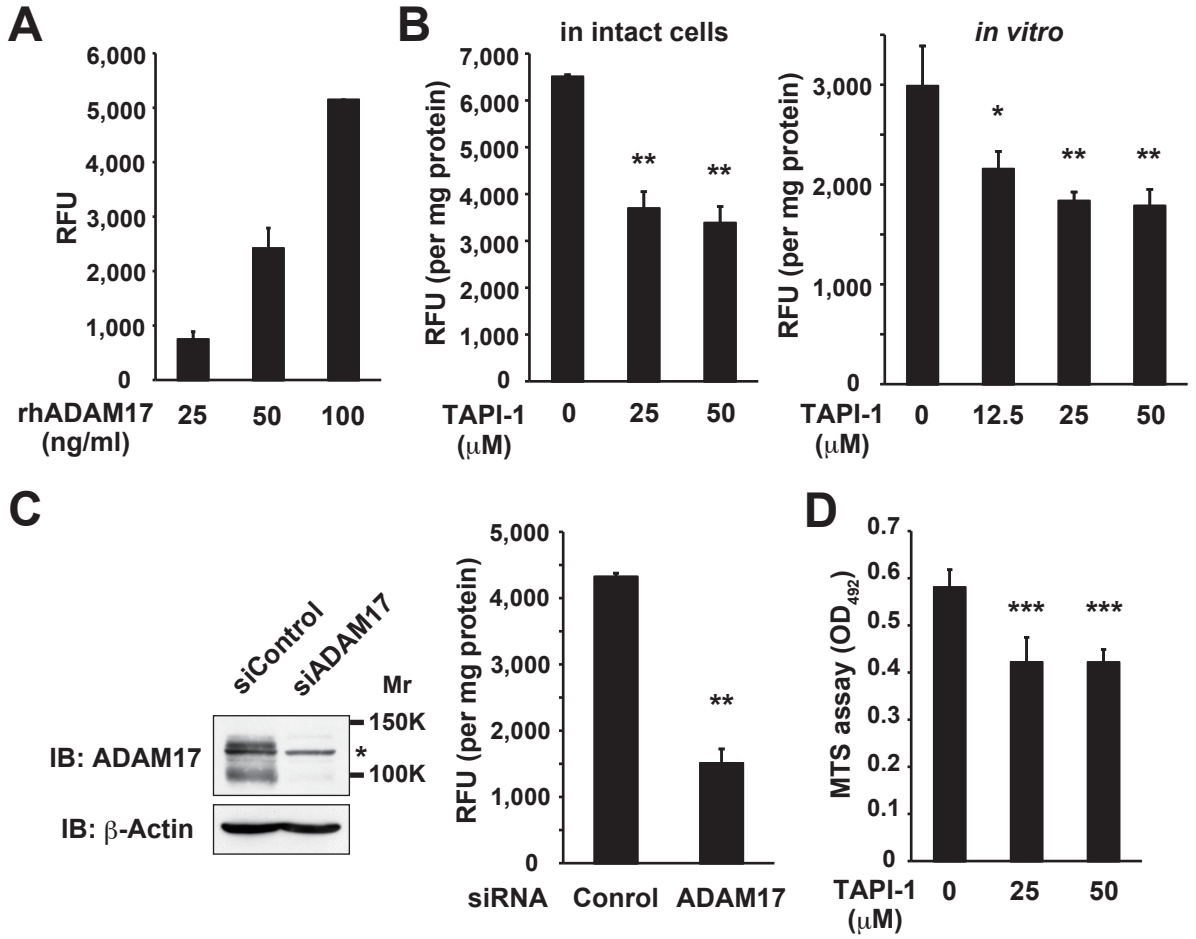

Figure S2 Effects of TAPI-1 treatment or ADAM17 RNAi on TACE protease activity in gastric cancer cells.

(**A**) Increasing doses of recombinant human ADAM17 protein were subjected to TACE activity analysis. (**B**) TAPI-1 suppresses the TACE activity of TMK-1 cells both in intact cells and in vitro. Left panel, TMK-1 cells were cultured in the presence of the indicated concentrations of TAPI-1 for 1 h (final DMSO concentration was 0.25%). TACE activities of the extracted cell lysates were analyzed. Right panel, cell lysates were prepared from TMK-1 cells without using protease inhibitors. Cell lysates were incubated with the increasing doses of TAPI-1 at 4 ° C for 1 h, followed by the TACE activity assay. (**C**) Left panel, TMK cells were transfected with control or ADAM17 siRNA. At 72 h after tranfection, cell lysates were subjected to Western blotting. Asterisk, non-specific band. Right panel, TACE activity assay was performed using TMK-1 cell lysates transfected with control or ADMA17 siRNA. (**D**) The MTS assay was performed after TMK-1 cells were left untreated or treated with the indicated concentrations of TAPI-1 for 30 h.

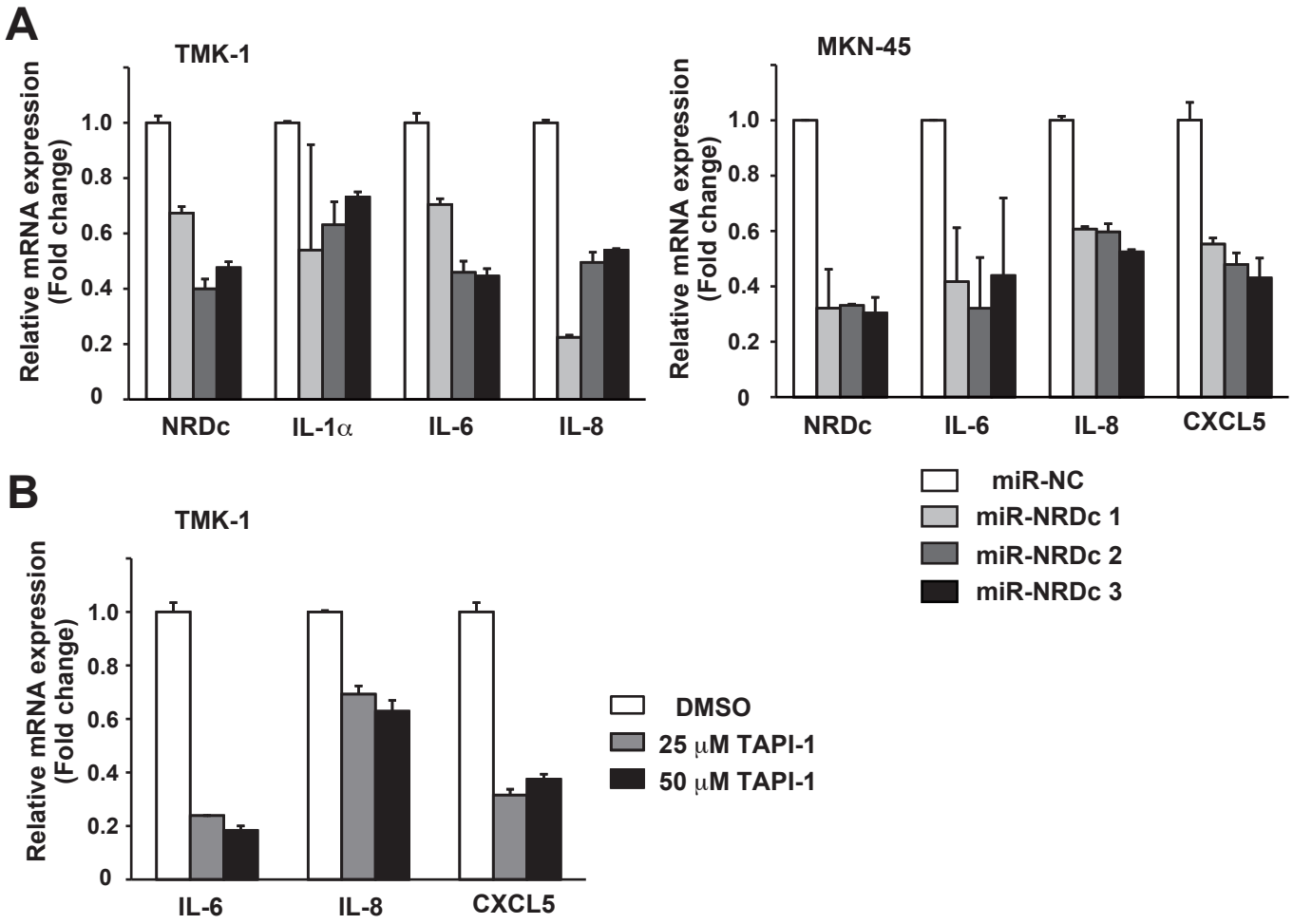

Figure S3 Changes in cytokine expressions after transient knockdown of NRDc or TAPI-1 treatment in gastric cancer cells.

(**A**) The control or each of the 3 different miR constructs targeting NRDc were transiently introduced into TMK-1 (left panel) or MKN-45 (right panel) cells. At 72 h after transfection, total RNA was extracted and qRT-PCR was performed for the indicated genes. (**B**) TMK-1 cells were left untreated or treated with 25 or 50 μM of TAPI-1 for 24 h. Then, mRNA levels were quantified by RT-PCR.

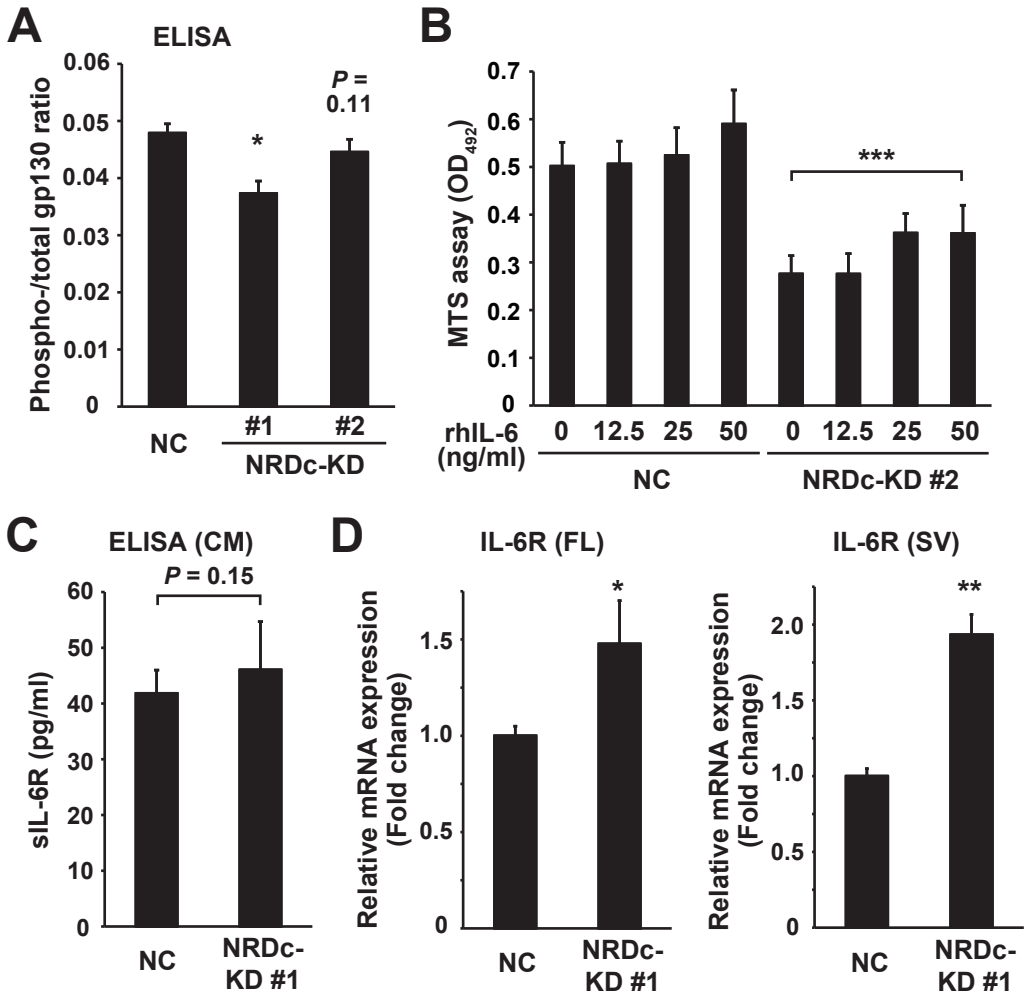

Figure S4 NRDc regulates gastric cancer cell growth through activation of autocrine IL-6–STAT3 signaling.

(**A**) Protein concentration of total or tyrosine-phosphorylated gp130 in each cell lysates was quantified by ELISA, respectively. The graph represents the ratios of phosphorylated form to total gp130. (**B**) The control or NRDc-KD #2 TMK-1 cells were cultured with the indicated concentrations of the recombinant human IL-6 protein for 24 h. The number of viable cells was quantified using the MTS assay. (**C**) Concentration of soluble IL-6 receptor (sIL-6R) in CM from the NC or NRDc-KD #1 TMK-1 cells was quantified by ELISA. Data are mean ± SD of triplicate assays from 2 independent samples. (**D**) mRNA levels of full-length (FL) and splice variant (SV) form of IL-6 receptor (IL-6R) in the NC or NRDc-KD #1 TMK-1 cells were analyzed by qRT-PCR.

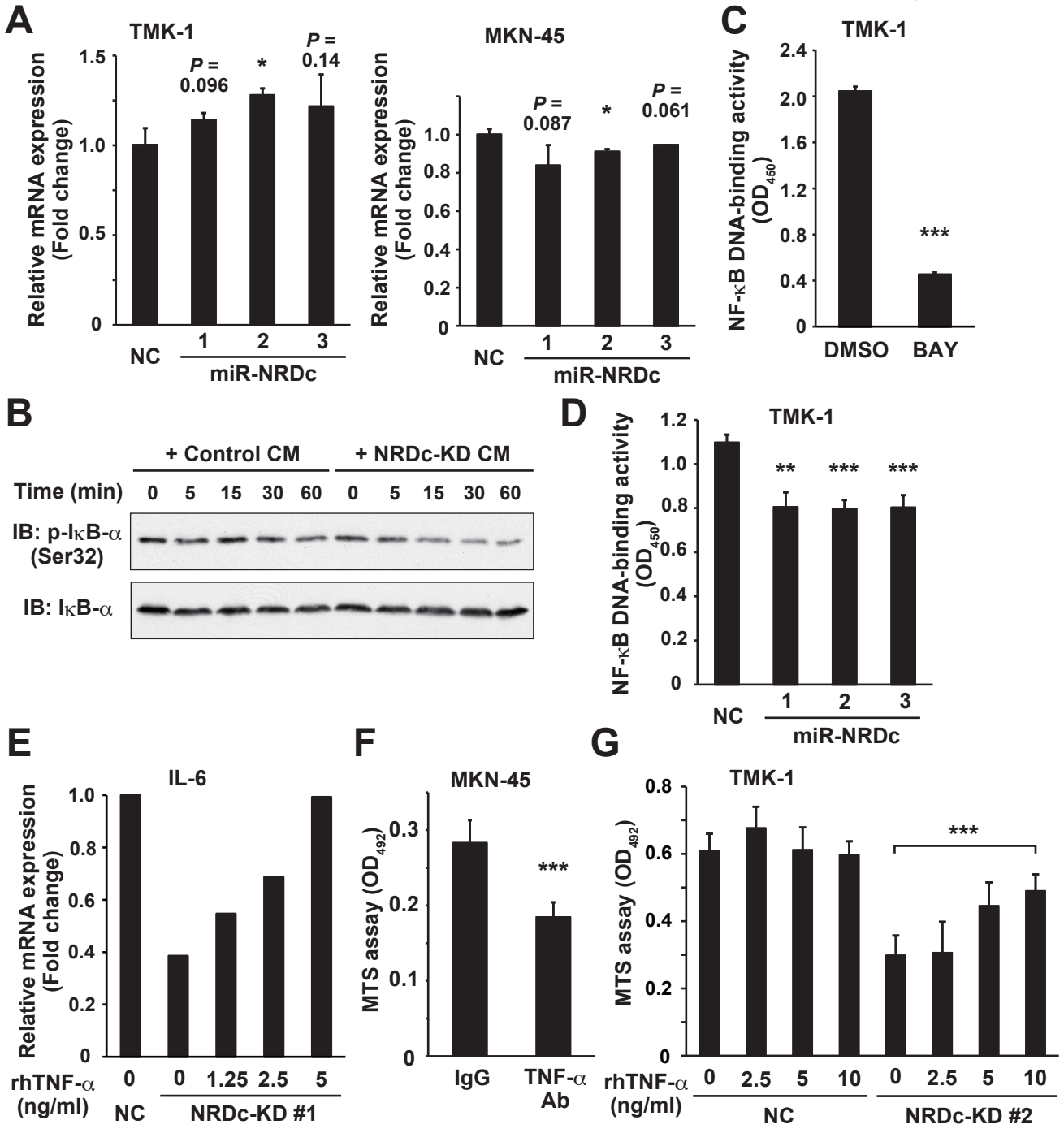

Figure S5 Reduced ectodomain shedding of TNF-α and attenuated NF-κB activity in NRDc-KD gastric cancer cells.

(**A**) Control or each of the NRDc-targeting miR constructs was transiently introduced into TMK-1 (left panel) or MKN-45 cells (right panel). At 72 h after transfection, TNF-α mRNA level was analyzed by qRT-PCR. (**B**) Serum-starved TMK-1 cells were incubated with CM from the NC or NRDc-KD #1 cells as in Fig 4A. Cell lysates at each time point were subjected to Western blotting with anti-phospho-IκB-α (Ser32) or anti-IκB-α antibody. (**C**) A confluent monolayer of TMK-1 cells was treated with 10 μM BAY 11-7082 or 0.01% DMSO for 1 h. Then nuclear extract was prepared, followed by the analysis with NF-κB p65 DNA-binding ELISA. (**D**) DNA-binding activity of NF-κB p65 in TMK-1 cells transiently transfected with the control or miR-NRDc constructs was measured. (**E**) The NRDc-KD #1 cells were incubated with the indicated concentrations of recombinant TNF-α for 3 h, and IL-6 mRNA expression was quantified by RT-PCR. (**F**) The MTS assay was performed after MKN-45 cells were cultured in the presence of 2 μg/ml control IgG or anti-TNF-α neutralizing antibody. (**G**) The control or NRDc-KD #2 TMK-1 cells were cultured with the indicated concentrations of the recombinant TNF-α protein for 24 h. The number of viable cells was quantified using the MTS assay.

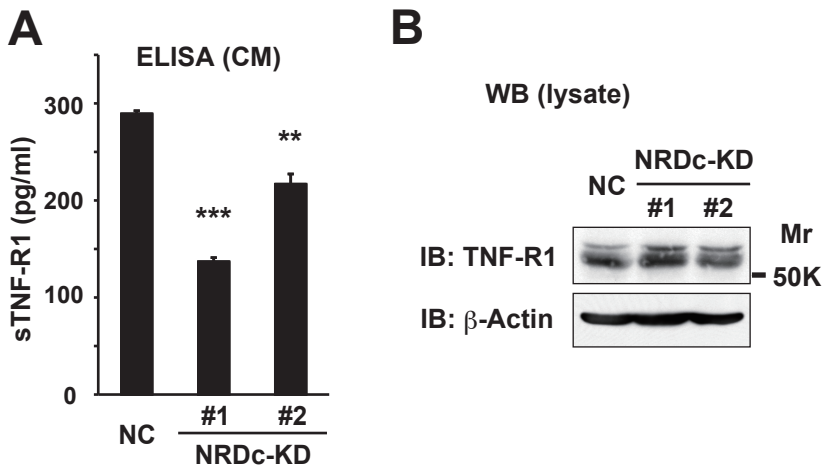

Figure S6 Changes in secretion of the soluble form of TNF receptor type 1 (sTNF-R1) after stable knockdown of NRDc in TMK-1 cells.
(**A**) Concentration of sTNF-R1 in CM from the control or NRDc-KD stable clones was quantified by ELISA. (**B**) Cell lysates prepared from the indicated cells were probed with anti-TNF-R1 or anti-β-actin antibody.

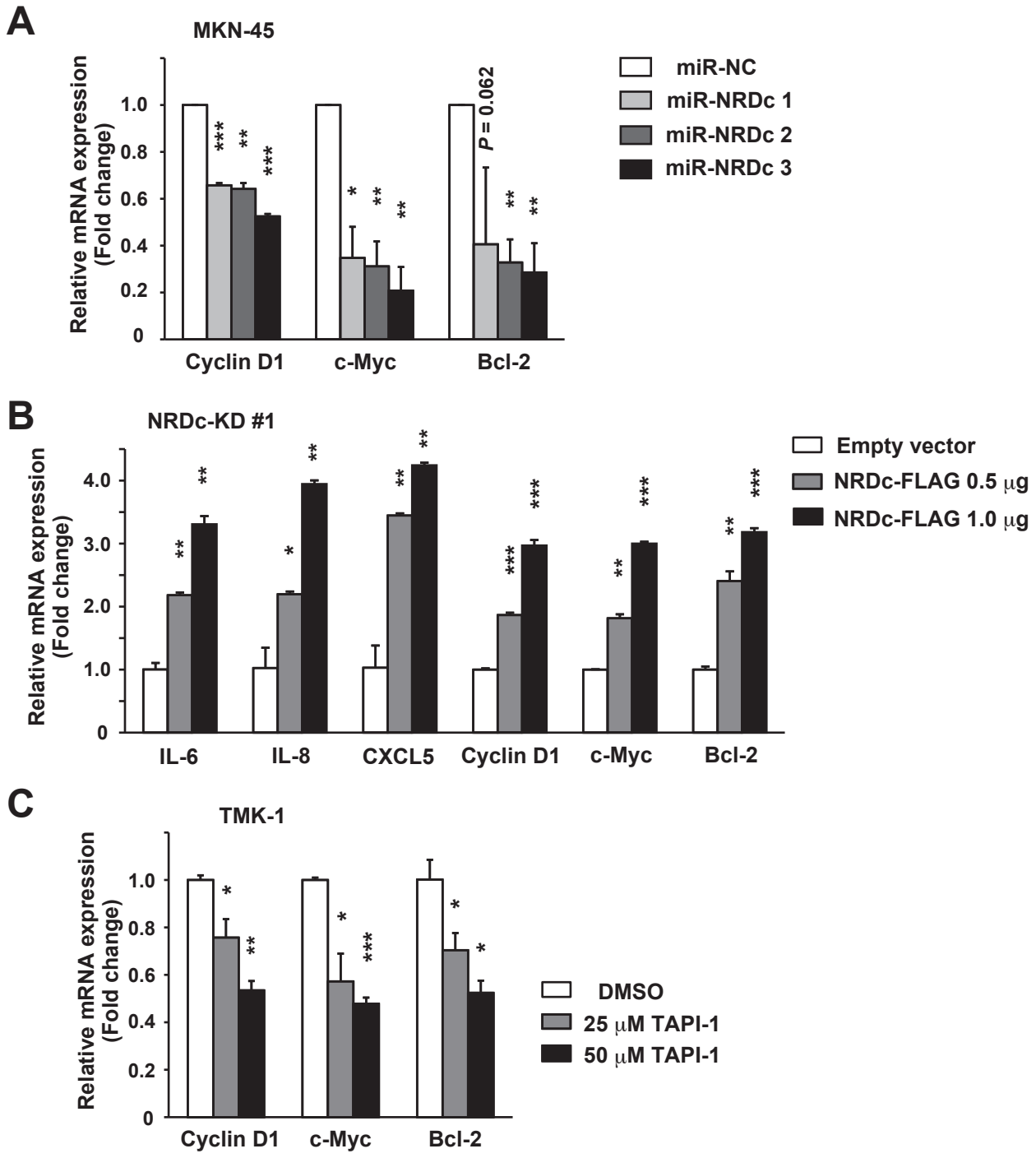

Figure S7 NRDc and ADAM proteases upregulate growth-related and anti-apoptotic genes downstream of STAT3 and NF-κB in gastric cancer cells.
(**A**) qRT-PCR analyses for cyclin D1, c-Myc, and Bcl-2 mRNA levels in MKN-45 cells into which NRDc-targeting vectors has been transiently introduced. (**B**) The empty vector or increasing doses of the NRDc-FLAG-expressing vector (RNAi-resistant form, total 1.0 μg of plasmid) was introduced into the NRDc-KD #1 cells grown in 60-mm diameter dishes as in Fig 2C. At 48 h after transfection, qRT-PCR was performed for the indicated genes. (**C**) qRT-PCR was performed using RNA extracted from TMK-1 cells treated with the indicated doses of TAPI-1 for 24 h.

Table SI
A list of used primers in RT-PCR experiments.

Gene	Forward	Reverse
human NRDc	5′-GGTCGGTGCGAAGACTCTG-3′	5′-AGATTCATCCGCTCCTAGACG-3′
human ADAM17	5′-GTGGATGGTAAAAACGAAAGCG-3′	5′-GGCTAGAACCCTAGAGTCAGG-3′
human ADAM10	5′-ATGGGAGGTCAGTATGGGAATC-3′	5′-ACTGCTCTTTTGGCACGCT-3′
human IL-1α	5′-ATCATGTAAGCTATGGCCCACT-3′	5′-CTTCCCGTTGGTTGCTACTAC-3′
human IL-6	5′-AAATTCGGTACATCCTCGACGG-3′	5′-GGAAGGTTCAGGTTGTTTTCTGC-3′
mouse IL-6	5′-TAGTCCTTCCTACCCCAATTTCC-3′	5′-TTGGTCCTTAGCCACTCCTTC-3′
human IL-6R (FL)	5′-CATTGCCATTGTTCTGAGGTTC-3′	5′-GTGCCACCCAGCCAGCTATC-3′
human IL-6R (SV)	5′-GCGACAAGCCTCCCAGGTTC-3′	5′-GTGCCACCCAGCCAGCTATC-3′
human IL-8	5′-ACTGAGAGTGATTGAGAGTGGAC-3′	5′-AACCCTCTGCACCCAGTTTTC-3′
human CXCL5	5′-TCCAAGGTGGAAGTGGTAGC-3′	5′-TTGTTTCCACCGTCCAAAAT-3′
human TNF-α	5′-CCTGTGAGGAGGACGAACAT-3′	5′-GGTTGAGGGTGTCTGAAGGA-3′
human cyclin D1	5′-GAACAAACAGATCATCCGCAAAC-3′	5′-GCGGTAGTAGGACAGGAAGTTG-3′
human c-Myc	5′-CCACAGCAAACCTCCTCACAG-3′	5′-GCAGGATAGTCCTTCCGAGTG-3’
human Bcl-2	5′-GGGGAGGATTGTGGCCTTC-3′	5′-CAGGGCGATGTTGTCCACC-3′
human 18S rRNA	5′-TAGAGTGTTCAAAGCAGGCCC-3′	5′-CCAACAAAATAGAACCGCGGT-3′
human GAPDH	5′-ATGGGGAAGGTGAAGGTCG-3′	5′-GGGGTCATTGATGGCAACAATA-3′
mouse GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′

SUPPLEMENTARY MATERIALS AND METHODS

Plasmids, antibodies, and reagents

pcDNA3/human NRDc (Met50-Lys1150)-FLAG and pME18S/FLAG-human pro-TNF-α were described previously (Hiraoka et al, 2008; Nishi et al, 2006). Anti-human NRDc mouse monoclonal antibodies were raised against recombinant NRDc (Nishi et al, 2006). Plasmid transfection experiments were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Anti-ADAM17 polyclonal antibody, anti-p44/42 MAPK (Erk 1/2) monoclonal antibody (137F5), anti-phospho-Erk 1/2 (Thr202/Tyr204) monoclonal antibody (D13.14.4E), anti-pan Akt monoclonal antibody (C67E7), anti-phospho-Akt (Ser473) monoclonal antibody (D9E), anti-STAT3 monoclonal antibody (124H6), anti-phospho-STAT3 (Tyr705) monoclonal antibody (D3A7), anti-TNF-α monoclonal antibody (D5G9), anti-TNF-R1 monoclonal antibody (C25C1), anti-IκB-α monoclonal antibody (44D4), and anti-phospho-IκB-α (Ser32) monoclonal antibody (14D4) were purchased from Cell Signaling Technology (Danvers, MA). Anti-FLAG M2 monoclonal antibody and anti-β-actin monoclonal antibody (AC-15) were obtained from Sigma-Aldrich (St. Louis, MO). Anti-human TNF-α, IL-6, and HB-EGF neutralizing antibodies, mouse IgG2a isotype control, and recombinant human TNF-α and IL-6 proteins were obtained from R&D Systems (Minneapolis, MN). HRP-conjugated anti-rabbit and mouse IgG antibody was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). Small molecule inhibitors, U0126, LY294002, AG490, and BAY 11-7082, were purchased from Merck/Calbiochem (Darmstadt, Germany), and TAPI-1 was obtained from Enzo Life Sciences (Farmingdale, NY). Other materials were obtained from commercial sources.

Sandwich ELISA for serum

To establish a sandwich ELISA system, all combinations of the 7 monoclonal antibodies for

NRDc were tested, and the optimum combination of clone #231 for coating and #304 for detection was determined. For measuring serum NRDc concentrations, an automated analyzer for the chemiluminescent enzyme immunoassay, SphereLight 180 (Olympus, Tokyo, Japan), was utilized according to the manufacturer's protocol. After optimization, human serum NRDc could be measured with high sensitivity and specificity, with a detectable limit of 50 pg/ml.

Immunohistochemistry

Human gastric cancer or mouse xenograft tumor tissues were fixed in 4% PFA for 24 h, transferred to PBS, and processed for embedding in paraffin. For immunostaining, 5 μm sections were incubated with the indicated primary antibodies (anti-NRDc mouse monoclonal antibody (#102, established in our laboratory), 1:1000; anti-phospho-STAT3 rabbit monoclonal antibody, 1:50) at room temperature for 2 h or at 4°C overnight. After rinsing with 5% washing solution, the specimens were reacted with biotinylated secondary antibody at room temperature for 1–2 h and incubated with the avidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) at room temperature for 30 min. Then, color was developed with diaminobenzidine solution (Dako, Glostrup, Denmark), followed by staining with hematoxylin.

Immunocytochemistry

Cells were fixed and permeabilized with 50% acetone/50% methanol. Non-specific staining was blocked with a blocking solution (5% donkey and 1% sheep serum in PBS). The cells were incubated overnight at 4°C with anti-NRDc mouse monoclonal antibody (#102: 5 μg/ml) and anti-ADAM17 (TACE) rabbit polyclonal antibody (H-300, Santa Cruz Biotechnology, CA: 5 μg/ml) in the blocking solution, followed by incubation with Alexa Fluor 488 anti-mouse IgG (Invitrogen/Molecular Probes) and Alexa Fluor 594 anti-rabbit IgG (4 μg/ml each) for 1 h at

room temperature. The stained cells were observed with a laser scanning confocal microscope (LSM 510 META; Carl Zeiss Microscopy, Germany).

Cell culture and preparation of CM

TMK-1, MKN-1, and MKN-45 human gastric cancer cell lines were described previously (Yokozaki, 2000). These cells were cultured in RPMI 1640 medium (Invitrogen/GIBCO) supplemented with 10% fetal bovine serum (growth medium). To prepare CM, confluent monolayers of the control or stable NRDc-KD TMK-1 cells were incubated in RPMI 1640 medium supplemented with 0.1% bovine serum albumin for 48 h. The cultured media were collected and centrifuged at 3,000 rpm at 4°C for 10 min, and the supernatant was harvested.

qRT-PCR

Total RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen) or RNeasy mini kit (Qiagen, Valencia, CA). cDNA generated by reverse transcription was subjected to real-time PCR assay using LightCycler 480 and FastStart Universal SYBR Green Master (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's protocols. mRNA levels of tested genes were normalized to 18S rRNA or GAPDH levels using a delta Ct method. Quantification of both full-length (FL) and splice variant (SV) forms of IL-6R mRNA was performed as previously reported (Nakanishi et al, 2004). Used forward and reverse primers are described in Table SI of Supporting information.

Western blotting

Cells were lysed with ice-cold 1× Nonidet P-40 (NP-40) lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol, and 1% NP-40) supplemented with protease and phosphatase inhibitor cocktails (from Sigma-Aldrich and Roche Applied Sciences, respectively), and the lysates were centrifuged at 15,000 rpm at 4°C for 15 min. Supernatants were collected and

mixed with the half amount of 3× Laemmli buffer supplemented with 6% 2-mercaptoethanol, followed by incubation at 95°C for 5 min. Aliquots were electrophoresed on an SDS-polyacrylamide gel and transferred to a Protran nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST) supplemented with 5% skim milk, and incubated with primary antibodies diluted in TBST, followed by incubation with HRP-conjugated secondary antibodies. Then, the blots were analyzed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA). If necessary, primary and secondary antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution (Toyobo, Osaka, Japan). Densitometric analyses were carried out using Image J software.

RNAi experiments

To knockdown endogenous NRDc expression in gastric cancer cells, we used the BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen). miRNA-expressing plasmids were constructed according to the manufacturer's protocol. Target sequences were as follows: miR-NRDc 1, 5′-GTTCTTCATCCACCCACTAAT-3′; miR-NRDc 2, 5′-CTGATGCAAACAGAAAGGAAA-3′; miR-NRDc 3, 5′-GAGAAATGGTTTGGAACTCAA-3′. We used the pcDNA6.2/EmGFP-miR-negative control vector (miR-NC from Invitrogen) as a negative control. It contains a non-targeting sequence for any vertebrate gene. To establish the clones stably expressing the miR-NRDc or control plasmid, TMK-1 cells transfected with the miR-NC or miR-NRDc 2 plasmid were cultured in the growth medium containing 5 μg/ml of blastcidin (Invitrogen). Several blastcidin-resistant clones were selected (if necessary, GFP-expressing cells were selected by FACS sorting) and maintained in the growth medium supplemented with the same concentration of blastcidin. For RNAi rescue experiments, the NRDc-expressing mutant plasmid was generated by introducing 4 silent mutations into the target region of miR-NRDc 2 using the inverse PCR method with the KOD-Plus-Mutagenesis

kit (Toyobo). pcDNA3/human NRDc (Met50-Lys1150)-FLAG (Nishi et al., 2006) was used as a template for PCR, and the primers used were as follows: forward primer, 5′-TCCGACGCGAATAGAAAGGAAATGTTGTTTGGA-3′; reverse primer, 5′-AGGCCTTGCAAGTTGATATTCACT-3′. The coding region of the mutant plasmid was verified by DNA sequencing.

For gene silencing of ADAM17 and ADAM10, we used the Stealth Select RNAi (Invitrogen) according to the manufacturer's instructions. Briefly, non-targeting negative control siRNA (12935-300, from Invitrogen) or siRNA duplex targeting ADAM17 (HSS110434) or ADAM10 (HSS100165) were introduced to TMK-1 cells grown in the growth medium without antibiotics (final siRNA concentration: 10 nM) using Lipofectamine RNAiMAX (Invitrogen).

In vitro cell proliferation analysis

Cell proliferation was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI). Briefly, 1.5 or 3.0 $\times$ 10^3 cells were seeded in 96-well plates and cultured in the growth medium containing 10% fetal bovine serum for 48–96 h (when indicated, cells were cultured for 24 h after seeding, followed by treatment with reagents such as neutralizing antibodies, small molecule inhibitors, or recombinant proteins for an additional 24 h). Then, 20 μl of MTS tetrazolium compound reagent was added to each well. After the plate was incubated at 37°C for 4 h in 5% CO_2 atmosphere, absorbance at 492 nm was measured using a plate reader. All experiments were performed in octuplicate, and results are shown as mean ± SD of values excluding the minimum and maximum ones.

TACE activity assay

Protease activity of ADAM17, also named as TACE, was analyzed using the InnoZyme TACE Activity Kit (Merck/Calbiochem), except for the substrate. The internally quenched fluorescent TACE substrate used was MCA-PLAQAV-Dpa-RSSSR-NH2 (R&D Systems). Its sequence is

derived from pro-TNF-α. After the substrate is cleaved by peptidases at the A-V amide bond, the fluorophore MCA is released from the quenching molecule Dpa. Briefly, cells grown in 10-cm dishes were lysed with ice-cold 1× NP-40 lysis buffer (not supplemented with any protease inhibitor) and centrifuged. One hundred microliters of cell lysates (protein concentration: 3 mg/ml) or recombinant human ADAM17 protein solution (for the positive control) were added to 96-well plates coated with anti-human ADAM17 antibody. After incubation at room temperature for 1 h, the plates were washed 5 times with the provided wash buffer. Then, 100 μl of 10 μM substrate solution was added to each well, and the plates were incubated at 37°C for 5 h. Fluorescence was measured using a fluorescence plate reader (excitation, 355 nm; emission, 460 nm).

Gene expression microarray analysis

Gene expression profiles were compared between the control and NRDc-KD TMK-1 cells using the Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) with the protocol of the Whole Transcript Sense Target Labeling Assay. Total RNA was extracted from the control or NRDc-KD #1 TMK-1 cells using the RNeasy Mini kit (Qiagen) and digested with DNase. Quality of the extracted RNA was analyzed using the Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). After hybridization with biotinylated sense-strand DNA generated using WT Sense Target Labeling and Control Reagent (Affymetrix), GeneChips were scanned using the GeneChip Scanner 3000 7G System. Microarray data were analyzed using the Affymetrix GeneChip Command Console Software (Affymetrix) and GeneSpring GX Version 10.0.2 (Agilent Technologies). The microarray data from this publication have been submitted to the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE29114.

Cytokine antibody array analysis and ELISA for CM

Cytokine expression profile in CM from the control or NRDc-KD stable clone was screened with RayBio Human Inflammation Antibody Array 3 (RayBiotech, Norcross, GA) according to the manufacture's protocol. The complete array map can be obtained at the manufacturer's Web site (http://www.raybiotech.com/map_all_m.asp#15). ELISA kits were purchased from R&D Systems (HB-EGF, IL-8, soluble IL-6R, soluble TNF-R1, total gp130, and phospho-gp130) and eBiosciences (TNF-α, human IL-6, and mouse IL-6) respectively, and experiments were performed according to the manufacturers' protocols.

STAT3 and NF-κB DNA-binding ELISA and reporter gene assay

DNA-binding activity of STAT3 or NF-κB p65 protein was quantified using the TransAM STAT3 or NFκB p65 Transcriptional ELISA Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 2–3 μg of nuclear extracts prepared from the cultured cells were added to a 96-well plate to which the oligonucleotide containing specific consensus sequence (for STAT3, 5′-TTCCCGGAA-3′; for NF-κB, 5′-GGGACTTTCC-3′) had been immobilized. After the plate was incubated at room temperature for 1 h, a solution of anti-STAT3 or anti-p65 antibody was added to each well and the plate was incubated for 1 h. Then, HRP-conjugated anti-IgG antibody was added to the wells, followed by incubation for 1 h. Then, substrate reagent was added and absorbance was read at 450 nm using the plate reader. For the NF-κB reporter gene assay, cells grown in 35-mm diameter dishes were cotransfected with 0.5 μg of pNF-κB-Luc (Clontech, Mountain View, CA) and 0.05 μg of pRL-TK (Promega). At 36 h after transfection, the cells were lysed and luciferase activities in the lysates were measured using the Dual-Luciferase Reporter Assay System (Promega).

Xenograft experiments

To analyze cell growth after xenograft transplantation, 1.0×10^6 of the WT, NC, or stable NRDc-KD TMK-1 cells were suspended in PBS and subcutaneously injected into the flanks of

nude mice (female, 6 weeks old). Tumor size was measured at 2, 3, and 4 weeks after inoculation. Tumor volume was calculated using the following formula: (length × width × depth)/2. Recipient mice were sacrificed at 4 weeks after inoculation, and xenotransplanted tumors were resected. Animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Kyoto University.

References

Nakanishi H, Yoshioka K, Joyama S, Araki N, Myoui A, Ishiguro S, Ueda T, Yoshikawa H, Itoh K (2004) Interleukin-6/soluble interleukin-6 receptor signaling attenuates proliferation and invasion, and induces morphological changes of a newly established pleomorphic malignant fibrous histiocytoma cell line. Am J Pathol 165: 471-480

Yokozaki H (2000) Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathol Int 50: 767-777