Supporting Information

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SI Materials and Methods

mESC, NPC, and MEF Cell Culture. R1E cells (ATCC) were maintained in 0.1% gelatin-coated plates with mESC growth medium containing knockout DMEM (KO-DMEM), 15% FBS, 100 mM MEM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine and 1× penicillin-streptomycin (Invitrogen) plus 1,000 units/mL of LIF (Millipore). Medium was changed every day and the cells were split every other day. Cells were kept at passage number lower than 15. R1E cells were treated with 0.5 μM adriamycin, 20 μM nutlin, or 25 joules/m^2 UV for 8 h. Neural progenitor cells were derived from R1E cells according to the protocol previously described (1). Briefly, embryoid bodies were formed in bacterial culture dish for 4 days before using ITSFn medium (DMEM/F12 containing 5 μg/mL insulin, 50 μg/mL transferrin, 30 nM selenium chloride, and 5 μg/mL fibronectin) to select nestin-positive cells for 7 days. Nestin-positive cells were then propagated in N2 medium (DMEM/F12 containing 25 μg/mL insulin, 50 μg/mL transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium chloride, 1 μg/mL laminin, 20 ng/mL bFGF, and 20 ng/mL EGF) for 1–4 passages. Mouse embryonic fibroblast cells were isolated from 13.5-day-old embryos and grown in DMEM containing 15% FBS.

ChIP-Chip Assay. ChIP assay was performed as described previously (2–4). ChIP-chip assay was done per Agilent's ChIP-chip protocol (version 10.0). Briefly, 50 million R1E cells were fixed with 1% formaldehyde at room temperature for 10 min. Fixation was quenched by adding glycine to a final concentration of 125 mM. Cell pellets were collected by centrifuging at 3,000 rpm for 5 min followed by washing with lysis buffer 1 (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100) and lysis buffer 2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). Precipitated chromatin was then resuspended in 3 mL of lysis buffer 3 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% N-lauroylsarcosine) and sonicated with a Misonix XL2020 for a total of 16 cycles of 30 s on and 1 min off. Fifty microliters of clear whole cell extract was saved as input. After immunoprecipitation with 10 μg of p53 antibody (M19, Santa Cruz) using Protein G Dynal beads (Invitrogen), reverse cross-linking was performed at 65° overnight. RNA and protein were digested with RNase A and proteinase K, respectively, and DNA was purified using the ethanol precipitation method. Ligation-mediated PCR was carried out to amplify the input and IP DNA. dUTP-Cy3 and -Cy5 dyes were used to label 2 μg of input and IP DNA, respectively. Five micrograms of labeled input and IP DNA were mixed and hybridized to a mouse promoter microarray (Agilent, G4490A) in an oven (SciGene) at 65° with rotation at 20 rpm. After washing, the microarrays were scanned with an Agilent DNA microarray scanner. Features were extracted with feature extraction software (Agilent, version 10.0). Data analysis was performed with DNA Analytics software (Agilent, version 4.0). The genomic binding sites of p53 were identified with a Whitehead per-array neighborhood mode.

Genomewide Location Analysis. Normalization of ChIP-Chip array data and detection of protein–DNA binding events were performed with Agilent DNA Analytics software (version 4.0). The processing steps included signal correction for nonspecific binding, dye-bias artifacts, and arraywide variations in intensity (median-based normalizations). The Whitehead per-array neighborhood model default heuristic was used to identify bound probes (triplicate probe set P value <0.001 and individual probe P values of one neighbor <0.005, or individual probe P value of the central probe <0.001 and individual P value of at least one neighbor <0.1). The maximum distance of 1,000 bp was set for two probes to be considered neighbors and for collapsing probes into bound regions. Probe chromosomal coordinates and gene annotation were used as provided by the DNA Analytics software (USCS mm8, NCBI Build 36).

Gene Expression Microarray and Analysis. Total RNA was isolated with an RNeasy mini kit (Qiagen). RNA quality was checked on an Agilent bioanalyzer. All samples used for microarray analysis had high quality score (RIN >9). Two hundred nanograms of RNA was reverse transcribed with random hexamer, amplified, and terminally labeled with biotin using an Affymetrix Whole Transcript Sense Target Labeling kit per manufacturer's protocol. Four replicates of each group were prepared, labeled, and hybridized to Affymetrix mouse Gene ST 1.0 GeneChip and scanned on Affymetrix GeneChip scanner 3000. Data were collected using Affymetrix GCOS software and analyzed in GeneSpring GX 9.0. The robust multiarray average (RMA) method and quantile normalization were carried out to produce gene expression summary measures. Differentially expressed genes were identified with one-way ANOVA and Tukey's post hoc comparisons. Genes were selected if the false discovery rate (FDR) adjusted P values were <0.05.

Pathway Analyses. The lists of bound genes and differentially expressed genes were tested for overrepresented pathways using DAVID collection of KEGG pathways (http://david.abcc.ncifcrf.gov/) (5). David's KEGG pathway analysis utilizes 94,739 pathways generated from 327 reference pathways. Fisher's exact test is used to test for pathway enrichment. Of note, DAVID made a modification in the test, which makes it more conservative.

Preparation of Conditioned Medium and Detection of the Antidifferentiation Activity. R1E cells were treated with UV at 25 joules/m^2 and cultured for 24 h in mESC growth medium (GM) without LIF. Floating cells were removed by centrifuging at 3,000 rpm for 5 min at 4°C. Clear supernatant was transferred to a new 15-mL tube and used as conditioned medium (CM). The medium generated from untreated cells was used as a control. To detect the antidifferentiation activity of CM, R1E cells were plated in 12-well plates and cultured in different combinations of conditioned medium and growth medium mixtures for 7 days. Specifically, CM was mixed with mESC GM without LIF (1:1). Both Wnt signaling inhibitors, sFRP2 and Dkk-1 (R&D Systems), were used at 250 ng/mL. Medium was changed every day and the cells were split every other day. Cells cultured in mESC medium with LIF were used as a undifferentiation control and without LIF as a differentiation control.

Immunostaining. R1E cells were fixed with 4% formaldehyde (vol/vol, in PBS) for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 15 min. Fixed cells were blocked in 10% goat serum (Sigma) in PBS at room temperature for 1 h, incubated for 2 h with Nanog antibody (Bethyl) at 1:200 dilution or Oct4 (Santa Cruz) at 1:100, washed three times with PBS before treated with Texas Red conjugated anti-rabbit IgG (H+L) (Vector Laboratories) at a 1:5,000 dilution. After washing with PBS three times, pictures of at least 10 random views were taken using an Olympus 1× 81 inverted fluorescence microscope. The total number of cells and number of Nanog- or Oct4-positive cells were counted.

Western Blotting. R1E cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) plus protease inhibitors and then sonicated with a Bioruptor (Diagenode). Lysate concentration was determined by Bradford (Biorad) or BCA (Thermo Scientific) assay. Twenty-five micrograms of total protein was loaded onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred onto a PVDF (Bio-Rad) membrane. The membrane was blocked with PBST (1× PBS with 0.05% Tween 20) + 5% milk at room temperature for 1 h and incubated with primary antibodies and then with horseradish peroxidase (HRP)-coupled secondary antibody, developed using SuperSignal West Dura Luminol/Enhancer solution (Thermo Scientific). Images were obtained using G:BOX (Syngene) imaging system. For multiple detections, membrane was stripped with Restore Plus Western Blot Stripping Buffer (Fisher Scientific). Antibodies used were: β-actin (Sigma, A3853); Nanog (Bethyl, A300-397a); Oct4 (Santa Cruz, sc-5279); p53 (Santa Cruz, sc-1312, M19); Wnt3 (Abcam, ab52568); anti-rabbit IgG (Jackson Immuno Research, 211–032-171); anti-mouse IgG (Jackson Immuno Research, 115–035-174); anti-goat IgG (Santa Cruz, sc-2350).

Reverse Transcription and Real-Time PCR. Total mRNA was isolated using an RNeasy mini kit (Qiagen). Reverse transcription was performed with a Multiscribe Reverse Transcriptase kit (Applied Biosystems). Real-time PCR was performed in triplicates with a total volume of 10 μL using a SYBR Green JumpStart Taq ReadyMix (Sigma). Reactions were run in a PCR System 7900HT (Applied Biosystems). Data were analyzed using SDS 2.3 software (Applied Biosystems) and then exported to an Excel file. Data normalization was done as described previously (2). Real-time PCR primers used in this study are listed in Table S4.

Short Interference RNA (siRNA) Transfection. mESCs were seeded in 12- or 6-well plates 1 day before transfection. Cells then were transfected twice with 100 nM siRNA using DharmaFECT 1 (Dharmacon) followed by 8-h adriamycin, nutlin, or UV treatment. Sequences of siRNAs are: p53 siRNA#1, sense, 5′-/5Phos/rCrCrA rCrCrArUrCrCrArCrUrArCrArArGrUrArCrArUrGT G-3′, anti-sense, 5′-rCrArC rArUrGrUrArCrUrUrGrUrArGrUrGrGrArUrG rGrUrGrGrUrA-3′; p53 siRNA#2, sense, 5′-/5Phos/rArCrCrArCrUrUrGrArUrGrGrArGrArGrUrArUrUrUrCrACC-3′, anti-sense, 5′-rGrGrUrGrArArArUrArCrUrCrUrCrCrArUrCrArArGrUrGrGrUrUrU-3′; Nanog siRNA#1, sense, 5′-GCAAUGGUCUGAUUCAGAAdTdT-3′, anti-sense, 5′-UUCUGAAUCAGACCAUUGCdTdT-3′; Nanog siRNA#2, sense, 5′-CGAGAACUAUUCUUGCUUAdTdT-3′, anti-sense, 5′-UAAGCAAGAAUAGUUCUCGdTdT-3′.

Alkaline Phosphatase (AP) Staining and Colony Survival Assay. AP staining was performed per manufacturer's protocol (Millipore, SCR004). Briefly, mESCs were cultured in 6-well plates for 5 days and fixed with 4% formaldehyde in PBS for 1–2 min. Cells were then washed once with 1× rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20) and stained with Fast Red Violet, naphthol, and water at ratio 2:1:1. For colony survival assay, 100–400 mESCs were seeded in 6-well plates precoated with 0.1% gelatin and then grown for 5 days in different combinations of growth medium and conditioned medium. The number of survived colonies was counted for each sample and the survival rate was calculated.

1. Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD (1996) Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* 59:89–102.
2. Huang J, et al. (2006) Repression of p53 activity by Smyd2-mediated methylation. *Nature* 444:629–632.
3. Boyer LA, et al. (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122:947–956.
4. Huang J, et al. (2007) p53 is regulated by the lysine demethylase LSD1. *Nature* 449:105–108.
5. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57.

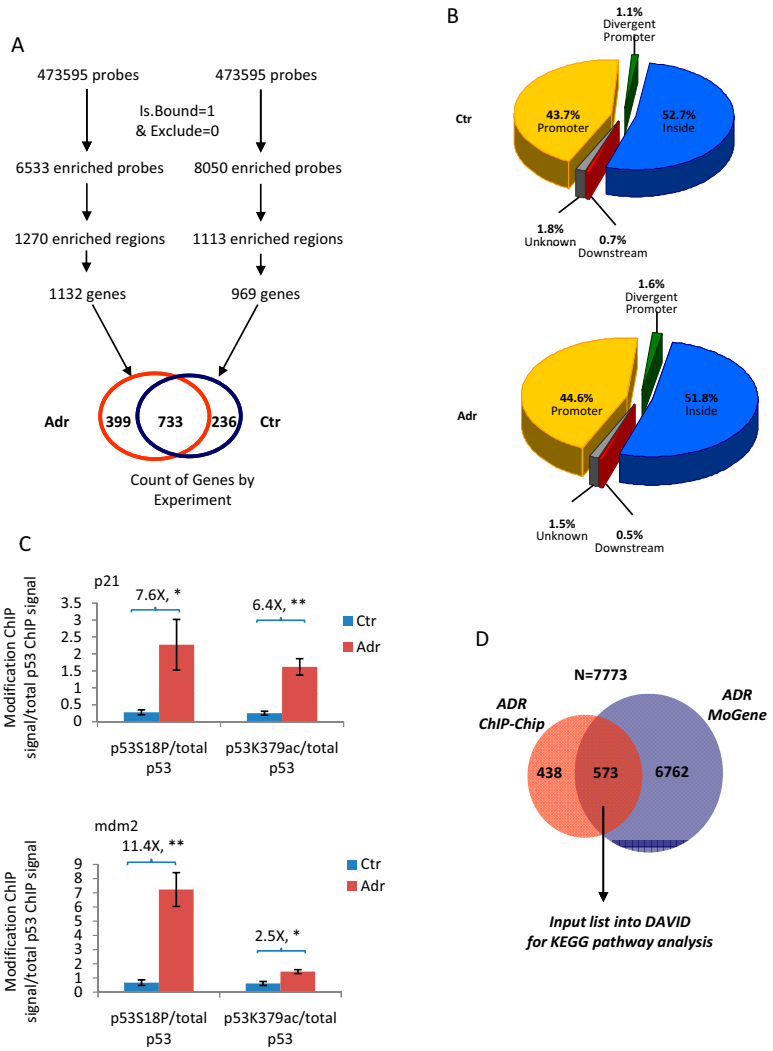

Fig. S1. (*A*) Schematic representation of ChIP-chip analysis workflow. Ctr, control or untreated; Adr, adriamycin treated. (*B*) Mapping of the binding sites (probes) to transcript annotation. Most of the probes are bound inside a gene or its promoter region (99% and 98% of known genes mapped in mESCs untreated, Ctr, or treated with adriamycin, Adr, respectively). Probes within the −5.5 kb and +2.5 kb genomic regions for all enriched genes were used to generate the pie charts. (*C*) p53 is activated after adriamycin treatment. Classical ChIP assays were performed using total p53, p53 Ser18 phosphorylation (p53S18P), and p53 Lys379 acetylation (p53K379ac) antibodies (see Fig. 1*C*). Ratios of p53S18P/total p53 and p53K379ac/total p53 on p53 binding sites of *p21* (*Upper*) and *mdm2* (*Lower*) genes were calculated and shown as *y* axis. Numbers in the figure show the fold increases of adriamycin treatment versus untreated condition. Shown are mean ± SEM, $n \geq 3$; $^{*}P < 0.05$; $^{**}P < 0.01$. (*D*) Schematic of common genes from ChIP-Chip and gene expression microarray. In total, 15,587 genes are available in both ChIP-chip and gene expression microarray platforms. Among them, 1,011 genes have a transcription factor binding site (TFBS) when treated with ADR and 7,335 are differentially expressed (Adr versus Ctr). A total of 573 genes are the common genes on ChIP-chip and gene expression microarray.

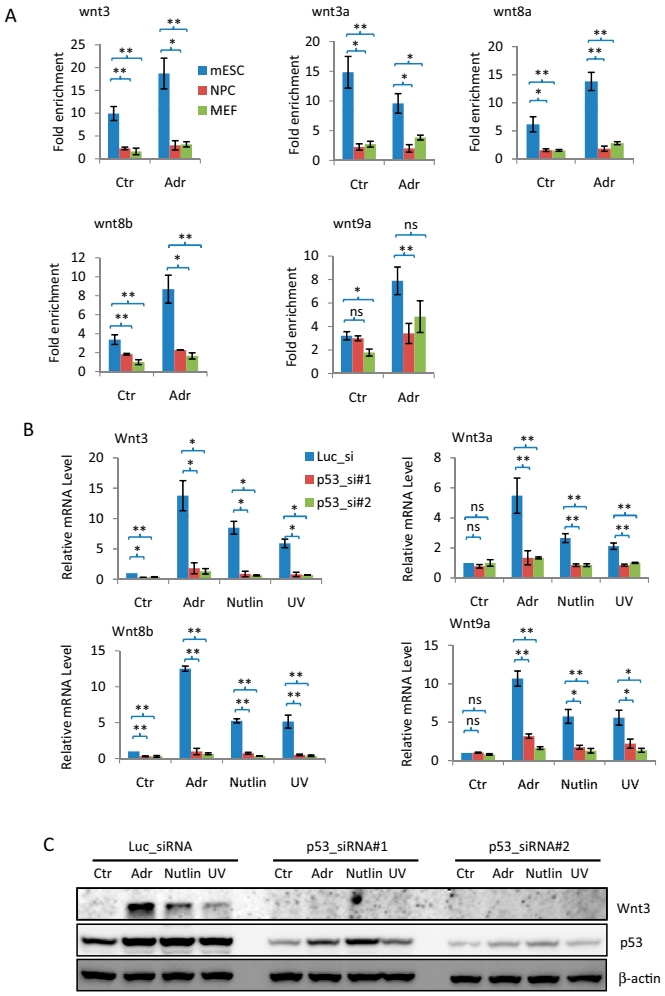

Fig. S2. (*A*) ChIP assays with p53 antibody on Wnt ligand genes in mESCs, NPCs, and MEFs. Shown are average fold enrichment (p53 ChIP signal versus IgG signal) ±SEM, $n \geq 3$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant. (*B*) The induction of Wnt genes is a p53-dependent stress response of mESCs. Real-time PCR analysis of Wnt ligand gene induction in mESCs transfected with *luciferase* siRNA (Luc_si), *p53* siRNA#1 (p53_si#1), or p53 siRNA#2 (p53_si#2) followed by being untreated (Ctr) or treated with adriamycin (Adr), nultin, and UV for 8 h. Shown are mean ± SEM, $n \geq 2$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant. (*C*) Western blot analysis of whole cell lysate of mESCs transfected with *luciferase* siRNA (Luc_si), *p53* siRNA#1 or p53 siRNA #2 followed by being untreated (Ctr) or treated with adriamycin (Adr), nultin, and UV for 8 h using Wnt3, p53, and β-actin antibodies.

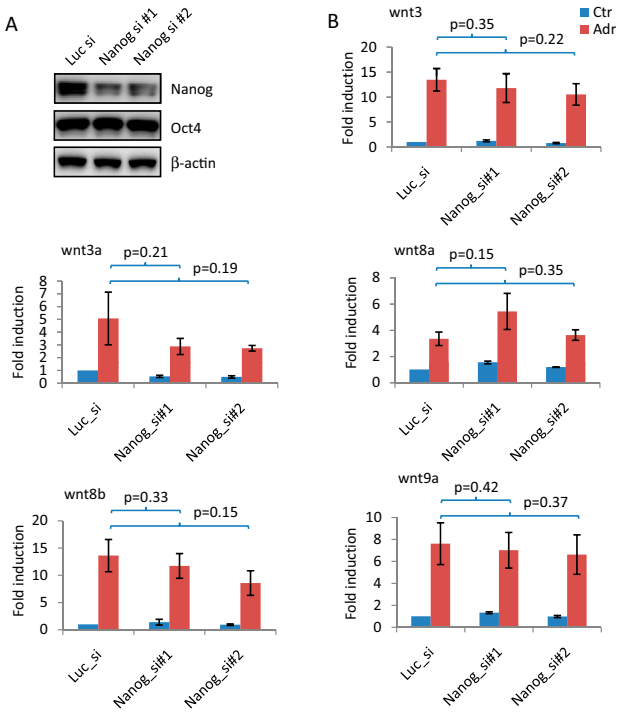

Fig. S3. Effect of Nanog knockdown on the induction of Wnt ligand genes in mESCs. (*A*) Western blot analysis of Nanog, Oct4, and β-actin. mESCs were transfected with luciferase siRNA (Luc_si), Nanog siRNA#1 (Nanog_si#1), and Nanog siRNA#2 (Nanog_si#2) for 2 days followed by adriamycin treatment for 8 h. (*B*) Real-time PCR analysis of the induction of Wnt ligand genes. Shown are mean ± SEM, $n = 3$; *P* values were shown in the figure.

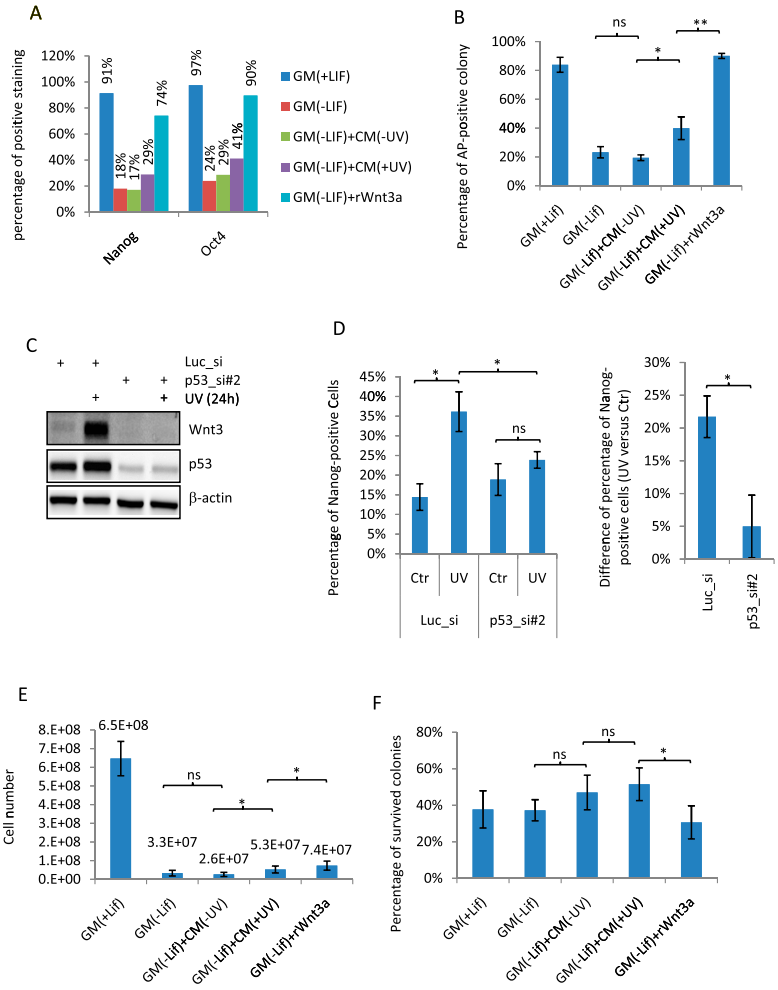

Fig. S4. Functional assays to detect antidifferentiation activity in conditioned medium (CM) from UV-treated mESCs. (*A*) Nanog- and Oct4-staining to measure the antidifferentiation activity in CM. mESCs were grown in growth medium (GM) or various combinations of GM and conditioned medium (CM). Cells were stained with Nanog and Oct4 antibodies. The percentages of Nanog- and Oct4-positive cells were counted. Recombinant Wnt3a was included as a positive control for this assay. (*B*) Alkaline phosphatase (AP) staining to detect the activity in CM of maintaining the self-renewal of mESCs. All of the assays were performed on day 7 after grown in different combinations of GM and CM. Shown are mean ± SEM, $n = 3$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant. (*C*) The antidifferentiation activity in CM is dependent on p53 status. Western blot analyses of Wnt3, p53, and β-actin. (*D*) (*Left*) Quantification of the percentage of Nanog-positive cells of mESCs grown in CM collected from mESCs transfected with *luciferase* siRNA or *p53* siRNA#2 (p53_si#2) untreated or treated with UV for 24 h. (*Right*) The difference of the percentage of Nanog-positive cells between mESCs grown in CM from UV-treated mESCs and cells in CM from untreated mESCs was shown for *luciferase* and *p53* siRNA transfections. Shown are mean ± SEM, $n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant. (*E*) Cell number counting and colony survival assay to measure the effects of CM from UV-treated mESCs on the proliferation and survival of mESCs. Cell number counting of mESCs grown under different conditions for 7 days as indicated. The number of initially seeded mESCs is 0.3 million. (*F*) Colony survival assay to assess the effect of various media on the survival of mESC colonies. Cells were grown in media for 7 days. Shown are mean ± SEM, $n = 3$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant.

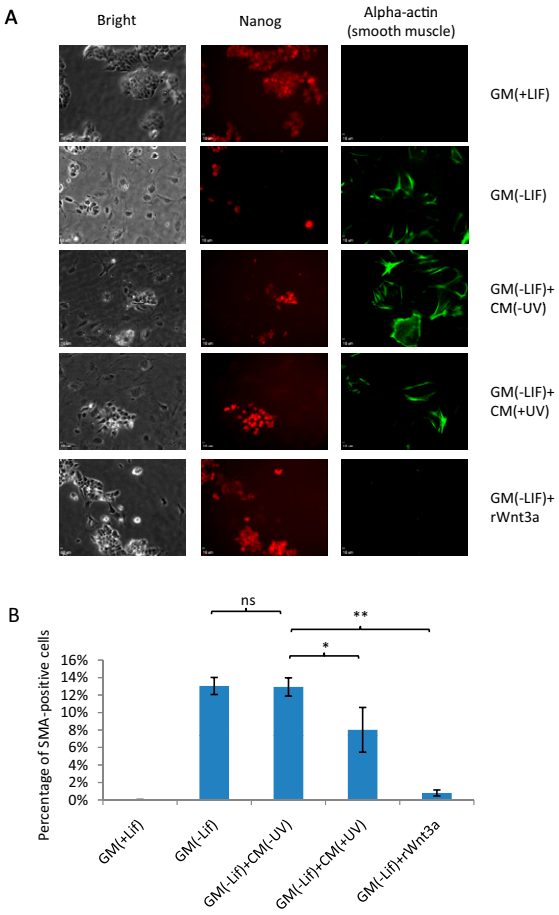

Fig. S5. Immunostainings of Nanog and SMA (smooth muscle α-actin) in mESCs grown in different combinations of growth medium and conditioned medium. (*A*) A representative staining of Nanog (pluripotent stem cell marker) and SMA (smooth muscle, mesoderm). (*B*) Percentage of SMA-positive cells. Shown are mean ± SEM, $n = 3$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)
Table S4 (DOC)