YMTHE, Volume *27*

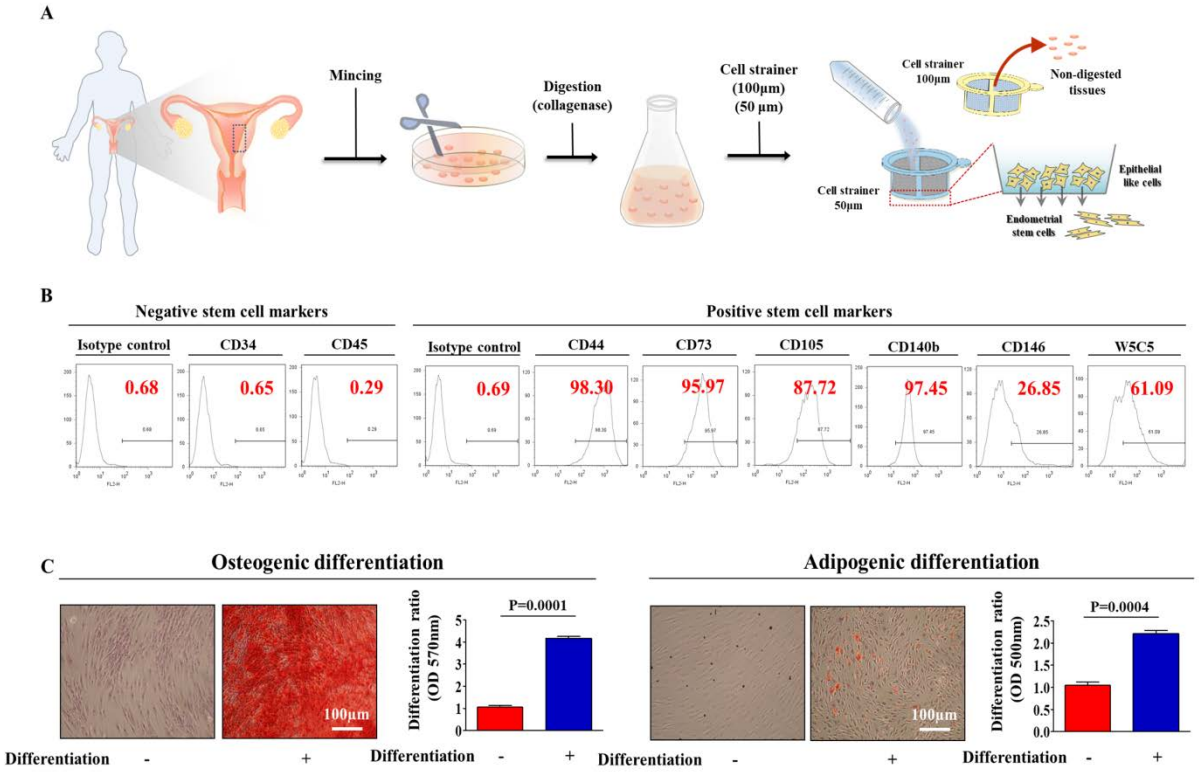
Supplemental Information

An Endogenous Anti-aging Factor, Sonic Hedgehog, Suppresses Endometrial Stem Cell Aging through SERPINB2

Ara Cho, Se-Ra Park, Soo-Rim Kim, Seungyoon Nam, Soyi Lim, Chan Hum Park, Hwa-Yong Lee, and In-Sun Hong

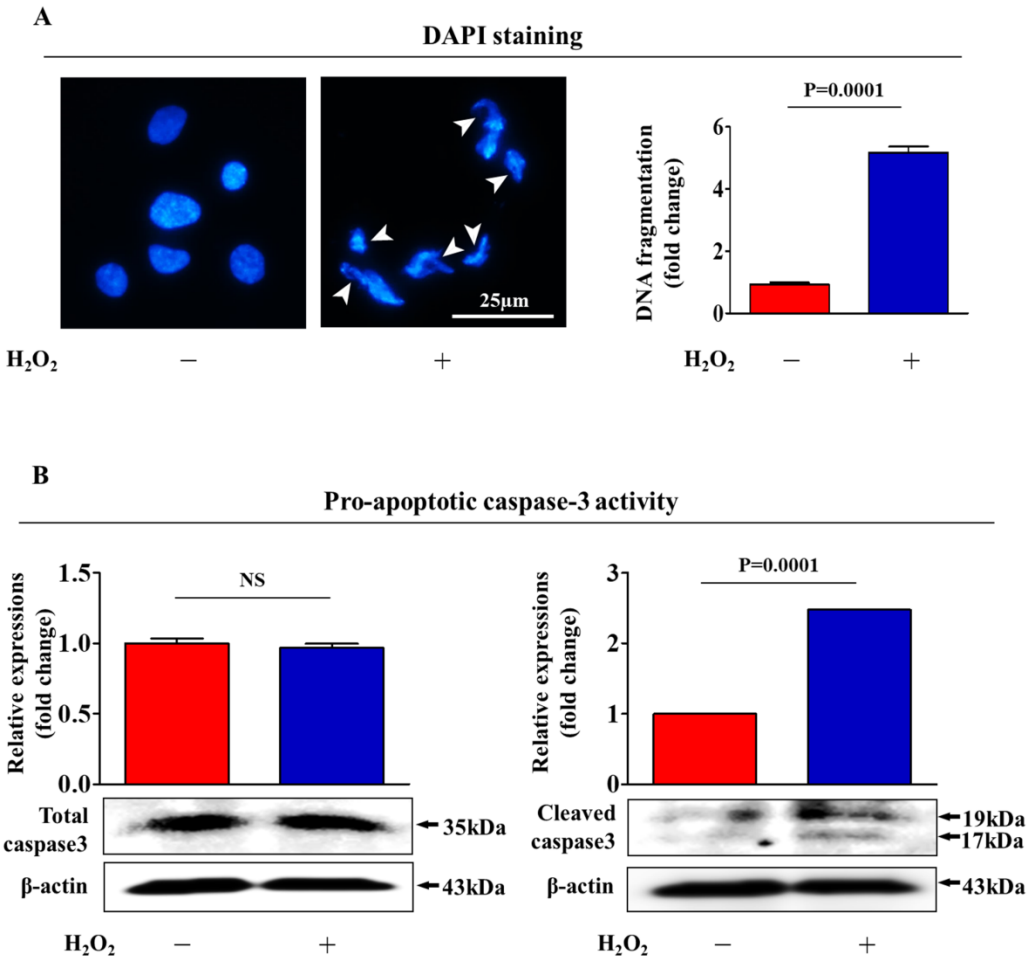
Supplementary figures and legends

Supplementary Figure 1



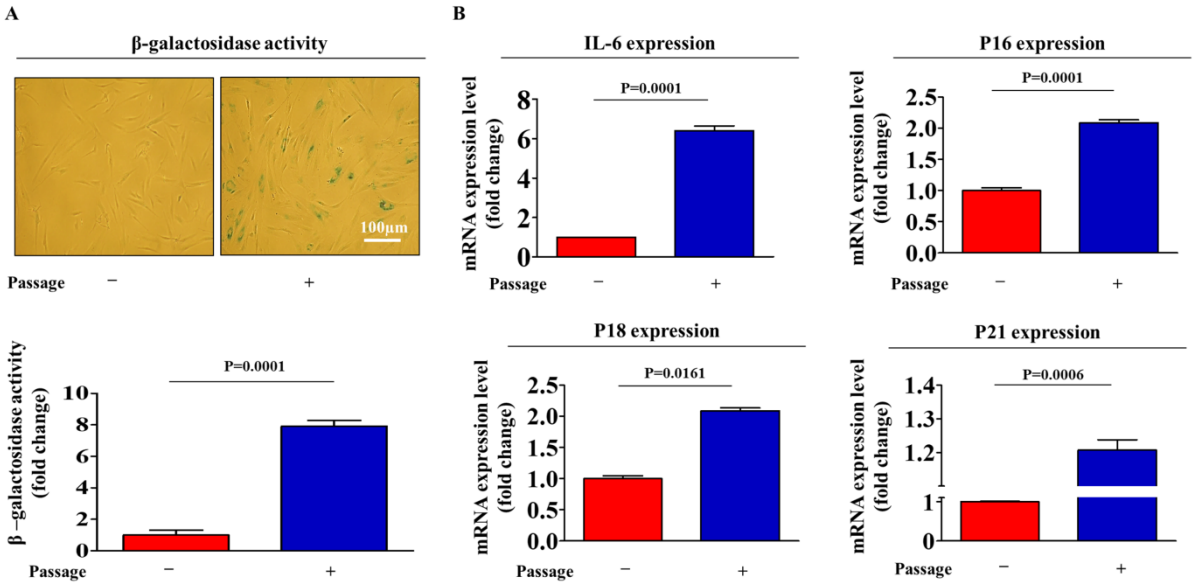
Supplementary figure 1. Isolation and characterization of human endometrial stem cells. Schematic representation showing the isolation procedures of human endometrial stem cells **(A)**. The isolated cells were positive for the stem cell markers CD44, CD73, CD105, and CD140b and negative for the hematopoietic markers CD34 and CD45 **(B)**. The capacity of these stem cells to differentiate into multiple lineages, specifically osteoblasts and adipocytes, was determined by alizarin red staining and oil red O staining, respectively. The relative quantification of calcium mineral content and lipid droplet formation was performed by measuring the absorbance at 570 nm and 500 nm, respectively **(C)**. The data are presented as the mean ± SD of three independent experiments.

Supplementary Figure 2



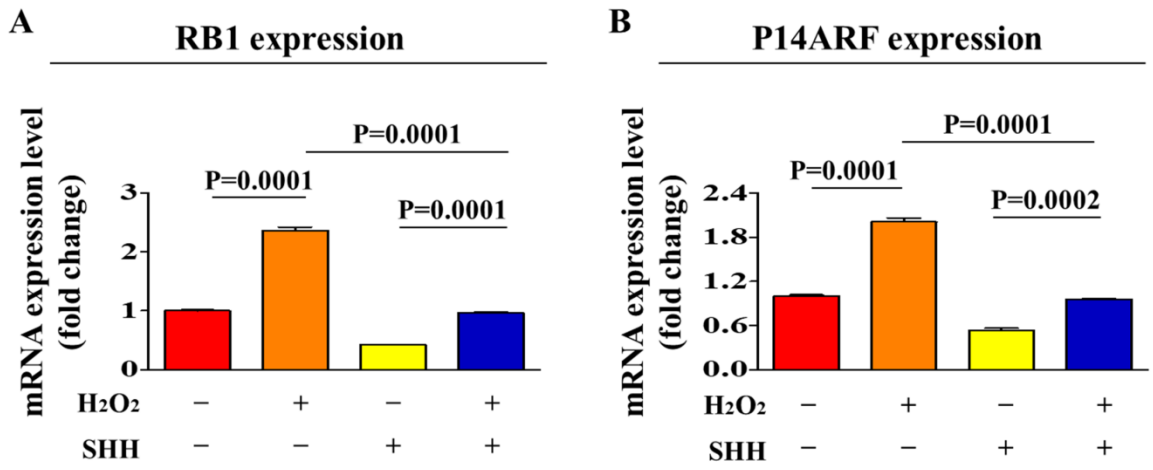
Supplementary figure 2. H_2O_2 treatment increased pro-apoptotic caspase 3 activity and subsequent DNA fragmentation. H_2O_2 treatment-induced apoptotic DNA fragmentation and condensation were visualized using DAPI staining **(A)**. Elevated levels of cleaved caspase-3 following GnRH treatment were assessed by western blotting **(B)**. DAPI staining was used to label the nuclei. β-actin was used as the internal control. The data are presented as the mean ± SD of three independent experiments.

Supplementary Figure 3



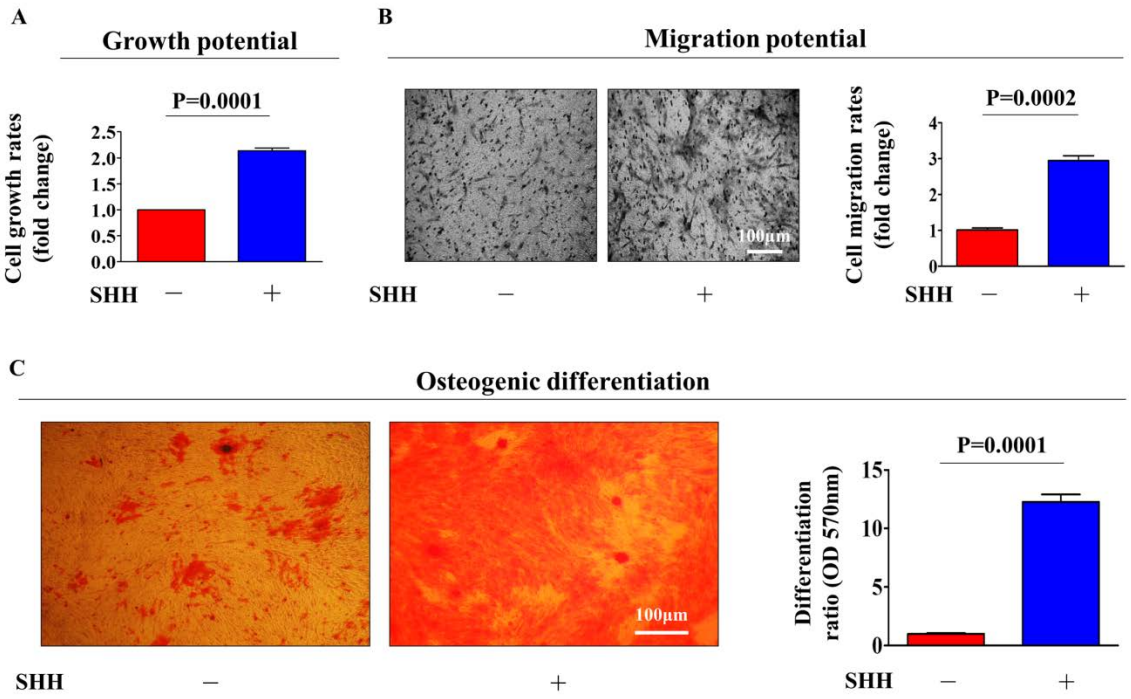
Supplementary figure 3. Replicative senescence commonly activates various senescence-associated phenotypes. Replicative senescence was induced by continuous subculture until passage 10; subsequent changes in stem cell aging were determined by measuring SA-β-Gal activity **(A)** and senescence markers such as IL-6, p16, p18, and p21 **(B)**. The data are presented as the mean ± SD of three independent experiments.

Supplementary Figure 4



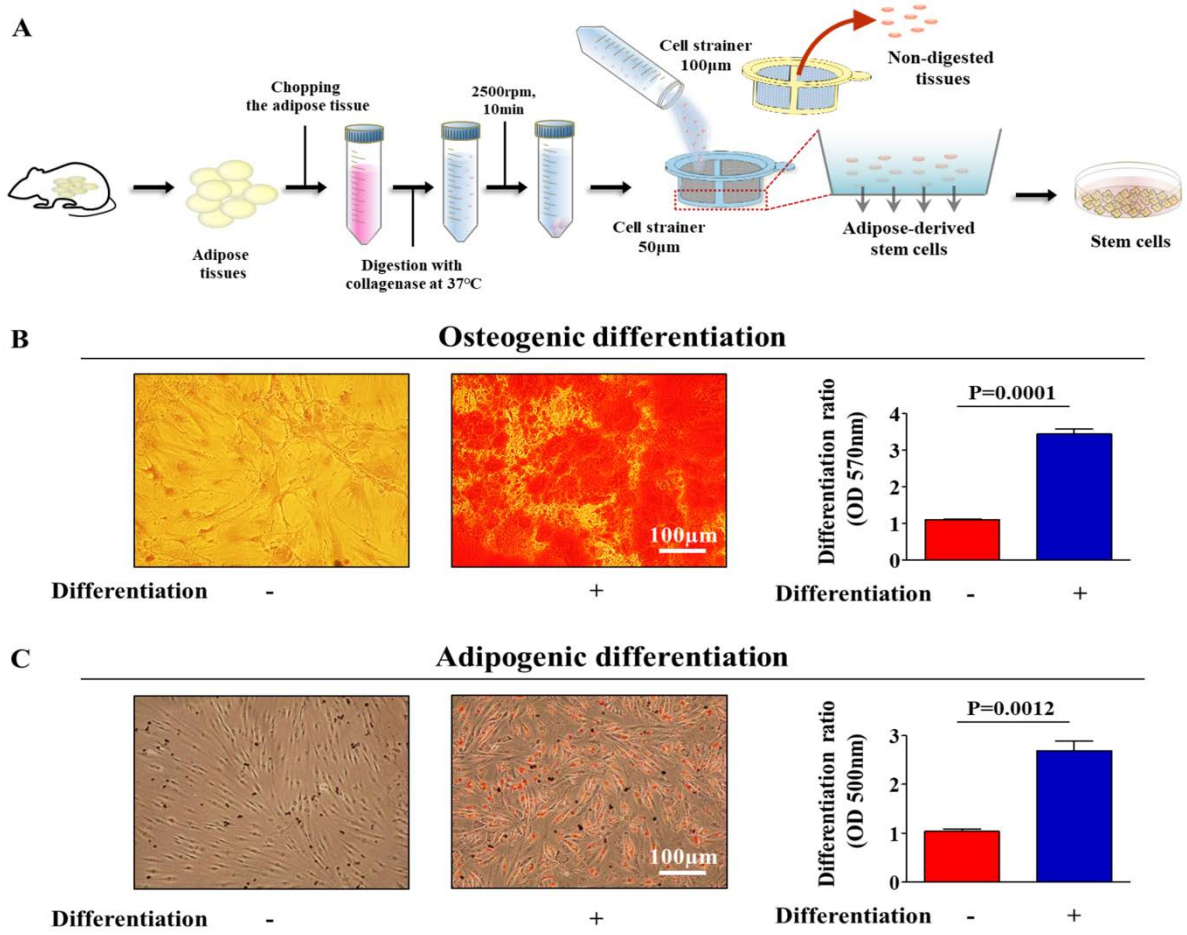
Supplementary figure 4. SHH attenuates oxidative stress-induced senescence marker expression. Oxidative stress-mediated senescence were induced by 700 nM hydrogen peroxide (H_2O_2) exposure for 1 h, and then endometrial stem cells were treated with or without SHH (4 μM). The ability of SHH to attenuate oxidative stress-induced senescence marker expression (RB1 and P14ARF) was determined by real-time PCR **(A-B)**. The data are presented as the mean ± SD of three independent experiments.

Supplementary Figure 5



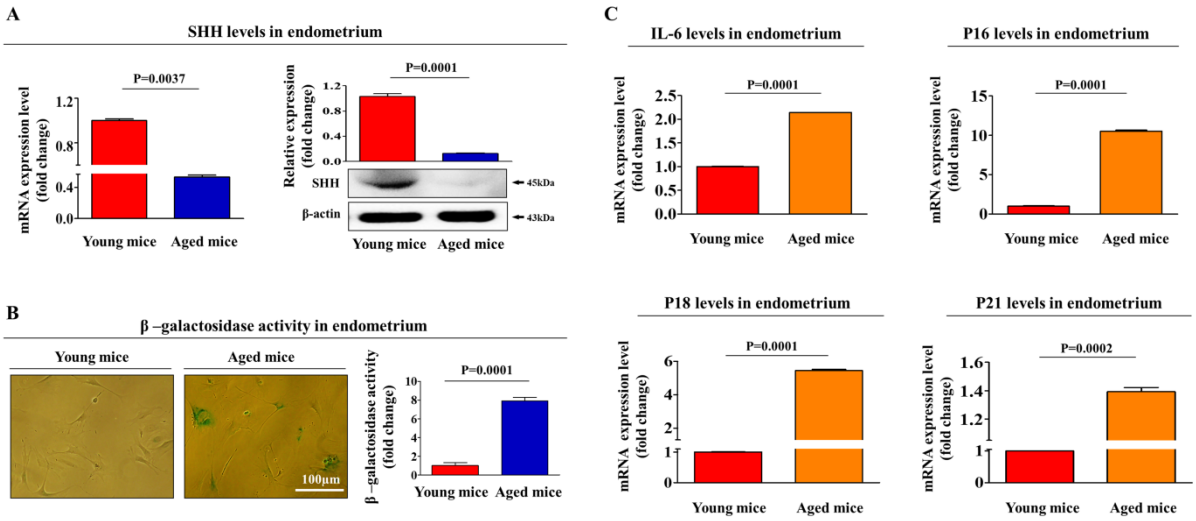
Supplementary figure 5. SHH promotes growth, migratory, and transdifferentiation capacity of endometrial stem cells *in vitro*. The stimulation of endometrial stem cell viability by SHH (4 μM) treatment for 72 h was determined by an MTT assay **(A)**. Endometrial stem cells were treated with SHH for 24 h, and the effect of SHH on the migration ability was then evaluated using a transwell migration assay. The SHH treatment significantly increased stem cell migration across the membrane compared with the negative control **(B)**. The effects of SHH on osteoblast differentiation were determined by alizarin red staining. The relative quantification of calcium mineral content was determined by measuring the absorbance at a wavelength of 570 nm **(C)**. The results are presented as the mean ± SD from three independent experiments.

Supplementary Figure 6



Supplementary figure 6. Isolation and characterization of mouse adipose tissue-derived stem cells. Schematic representation describing the isolation procedures of mouse adipose tissue-derived stem cells **(A)**. Their capacity to differentiate into multiple lineages, specifically osteoblasts **(B)** or adipocytes **(C)**, was determined by alizarin red and oil red O staining, respectively. The relative quantification of the calcium mineral content and lipid droplet formation was determined by measuring the absorbance at 570 nm and 500 nm, respectively. The data represent the mean ± SD from three independent experiments.

Supplementary Figure 7

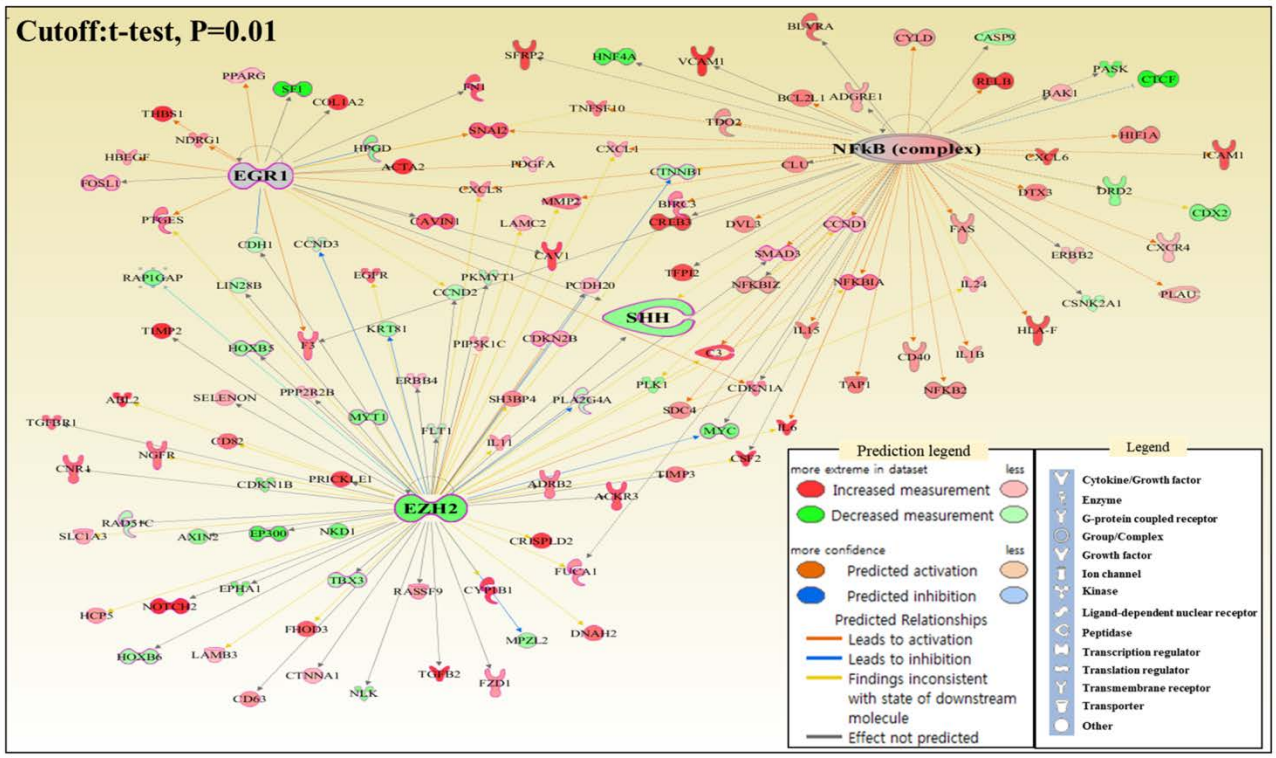


Supplementary figure 7. SHH expression was downregulated and replicative senescence commonly activates in endometrial stem cells derived from aged mice, recpectively. Endometrial stem cells were isolated from both young and aged endometrial tissues. Both mRNA and protein levels of SHH were evaluated using real-time PCR and western blotting, respectively **(A)**. The changes in stem cell aging were determined by measuring SA-β-Gal activity **(B)** and senescence markers such as IL-6, p16, p18, and p21 **(C)**. β-actin was used as an internal control. The data are presented as the mean ± SD of three independent experiments.

Supplement figure 8

A

Negative correlation between SHH-related pathways and cellular senescence

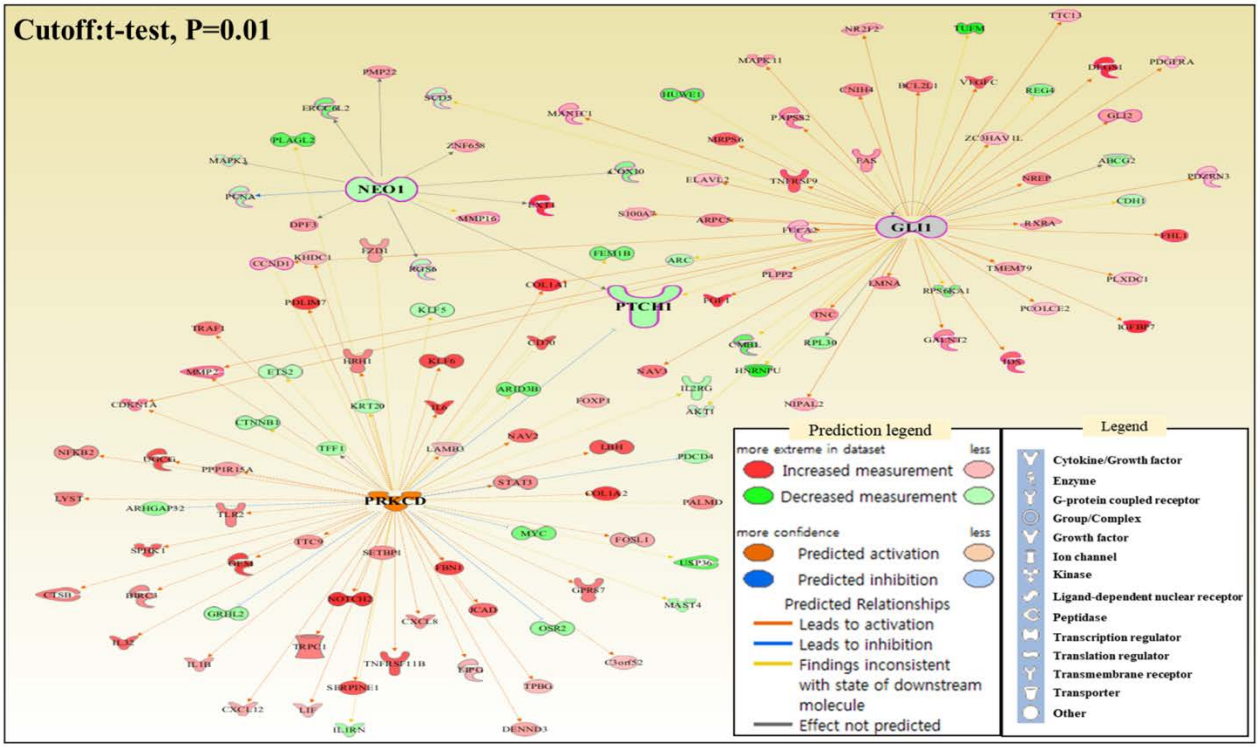


Signaling pathways	Predicted activation group	# of Target molecules in dataset	Predicted activation state	Activation z-score	P-value of overlap
NFKB	Non-senescent cells (vs Senescent cells)	113	Activation	5.081	1.86E-01
EGR1	Non-senescent cells (vs Senescent cells)	22	Activation	2.868	2.31E-01
EZH2	Non-senescent cells (vs Senescent cells)	82	Activation	3.065	3.17E-01

Supplement figure 8

B

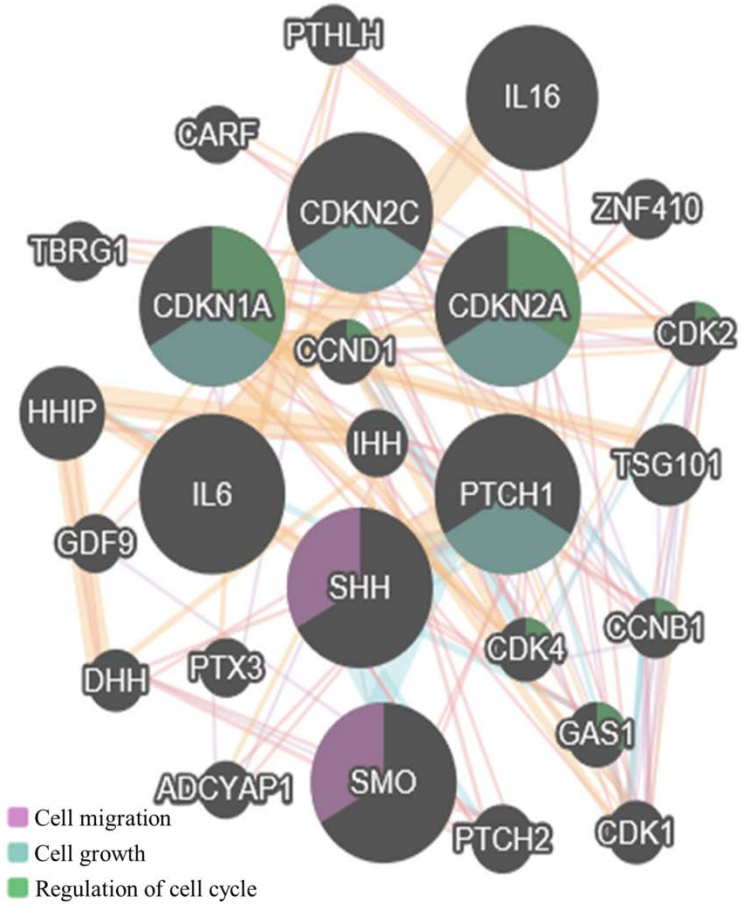
Negative correlation between PTCH1-related pathways and cellular senescence



Signaling pathways	Predicted activation group	# of Target molecules in dataset	Predicted activation state	Activation z-score	P-value of overlap
PRKCD	Non-senescent cells (vs Senescent cells)	70	Activation	4.161	1.23E-02
NEO1	Non-senescent cells (vs Senescent cells)	11	Not predicted		1.63E-02
GIL1	Non-senescent cells (vs Senescent cells)	52	Activation	3.428	1.00E00

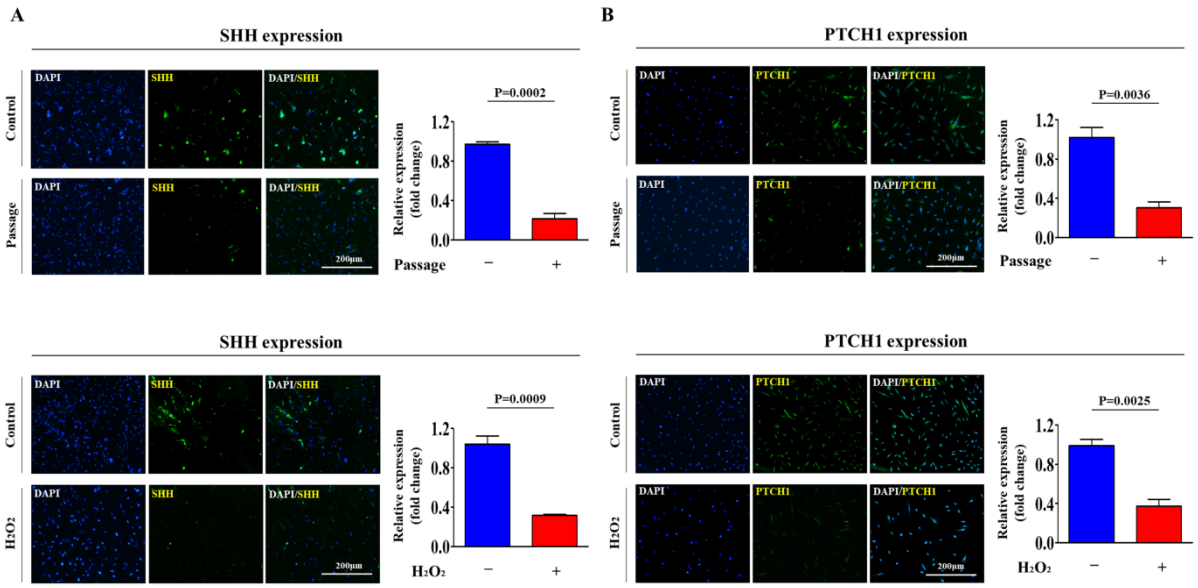
Supplement figure 8

C



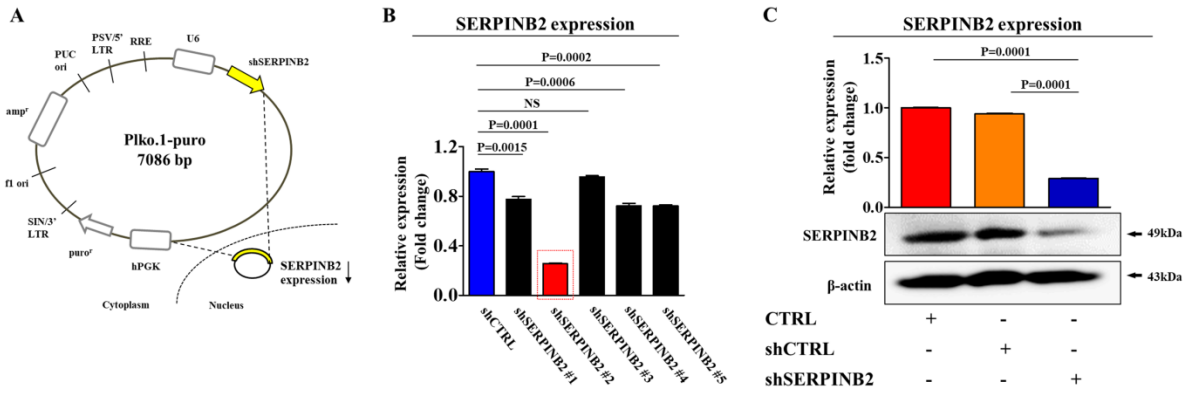
Supplemental Figure 8. Negative correlation between SHH- or PTCH1-related pathways and cellular senescence. Differentially expressed genes from nonsenescent proliferative cells and senescent cells (GSE32474) were applied to ingenuity pathway analysis (IPA) software (http://www.ingenuity.com) to predict the activation state (either activated or inhibited) of the SHH **(A)** or PTCH1 **(B)** level itself and its related signaling pathways. Clinical big data were analyzed using the Seiber dataset (GSE43996 and GSE9452) from 'R2: Genomics Analysis and Visualization Platform (http://r2.amc.ml)'. Signaling network analysis was performed using GeneMANIA (http://www.genemania.org) to predict the connections between SHH or PTCH1 and cell growth or migration **(C)**.

Supplementary Figure 9



Supplemental Figure 9. The expression of SHH and its receptor PTCH1 was significantly decreased in senescent stem cells. Replicative and oxidative stress-mediated senescence were induced by continuous subculture until passage 10 and 700 nM hydrogen peroxide (H_2O_2) exposure for 1 h, respectively. Cells were stained with antibodies that were specific for SHH **(A)** or PTCH1 **(B)**. DAPI staining was used to label the nuclei within each field. The results represent the means ± SD from three independent experiments.

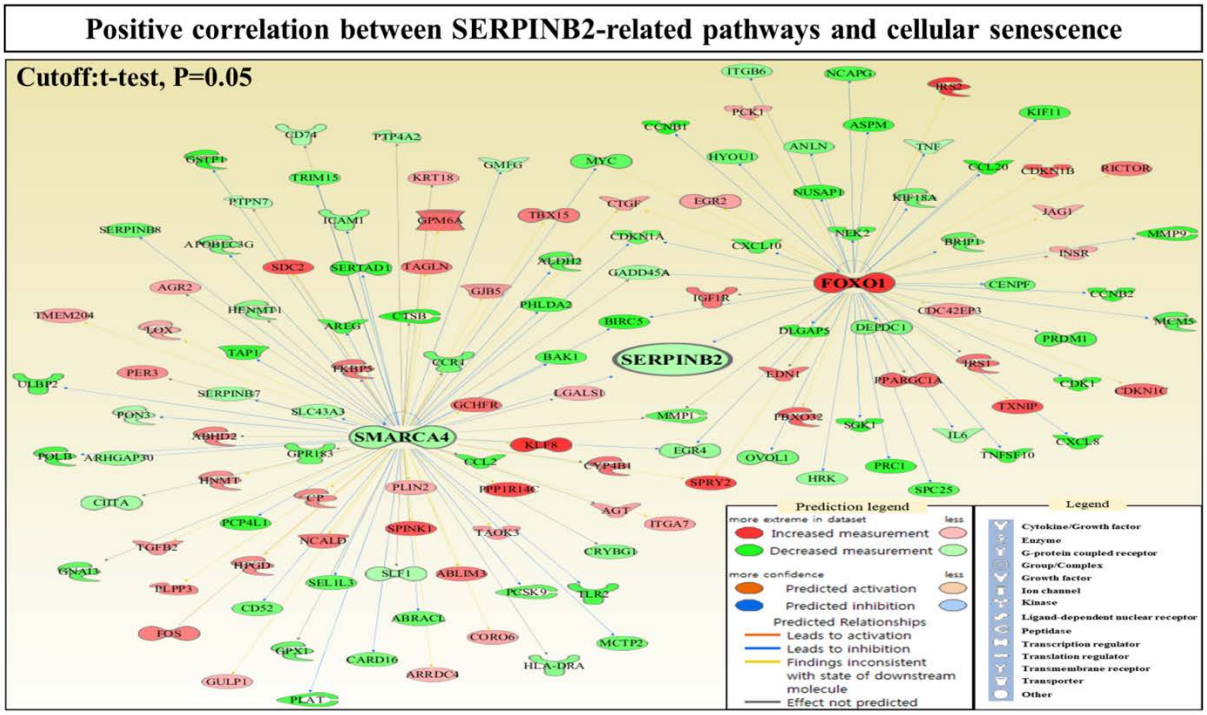
Supplementary Figure 10



Supplemental Figure 10. Knockdown efficiency of shRNAs targeting SERPINB2. Endometrial stem cells were stably transduced with shRNA, which targets SERPINB2, or with a nontargeting control shRNA **(A)**. Successful knockdown of SERPINB2 was verified based on the RNA **(B)** and protein **(C)** levels in endometrial stem cells. β-actin was used as the internal control. The results represent the mean ± SD from three independent experiments.

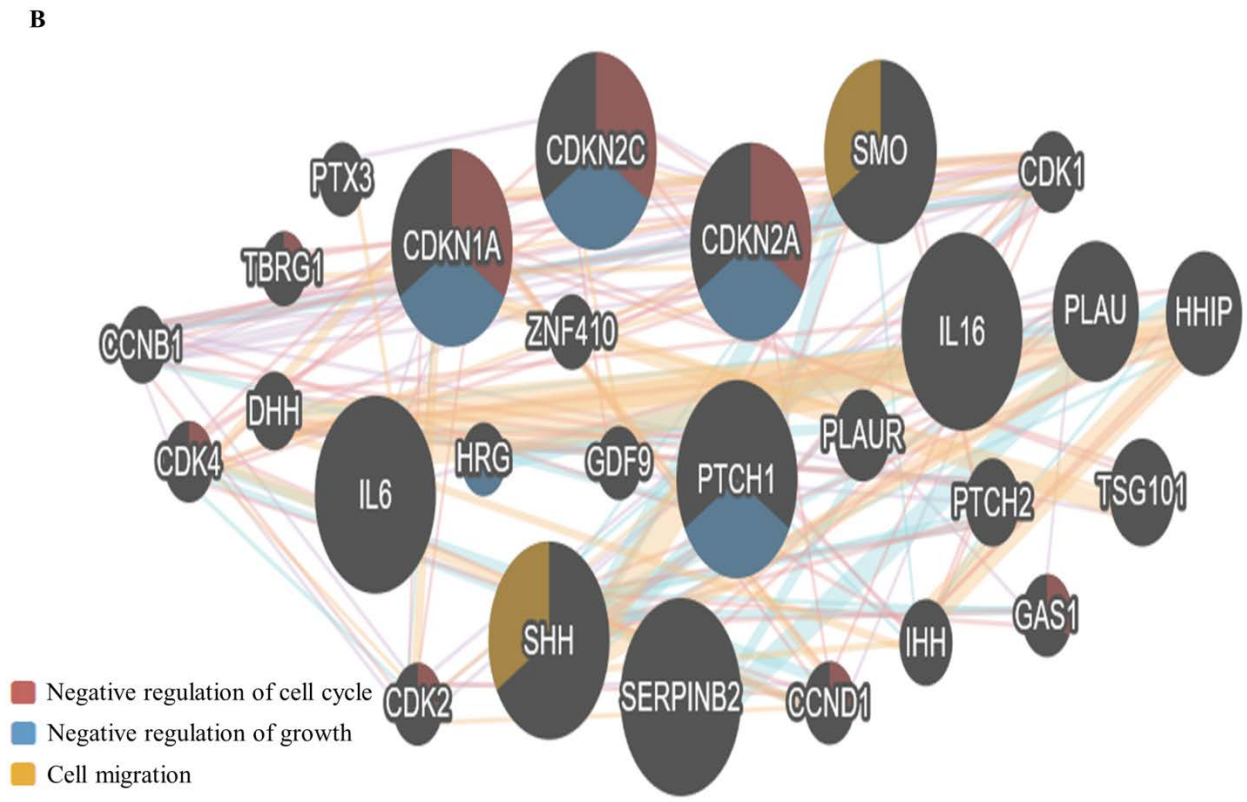
Supplement figure 11

A



Signaling pathways	Predicted activation group	# of Target molecules in dataset	Predicted activation state	Activation z-score	P-value of overlap
SMARCA4	Non-senescent cells (vs Senescent cells)	213	Inhibition	-2.536	4.37E-01
FOXO1	Non-senescent cells (vs Senescent cells)	55	Inhibition	-2.813	4.28E-02

Supplement figure 11



Supplemental Figure 11. Positive correlation between SERPINB2-related pathways and cellular senescence. Differentially expressed genes from nonsenescent proliferative cells and senescent cells were applied to ingenuity pathway analysis (IPA) software (http://www.ingenuity.com) to predict the activation state (either activated or inhibited) of the SERPINB2 level itself and its related signaling pathways **(A)**. Signaling network analysis was performed using GeneMANIA (http://www.genemania.org) to predict the connections between SERPINB2 and cell growth or migration **(B)**.