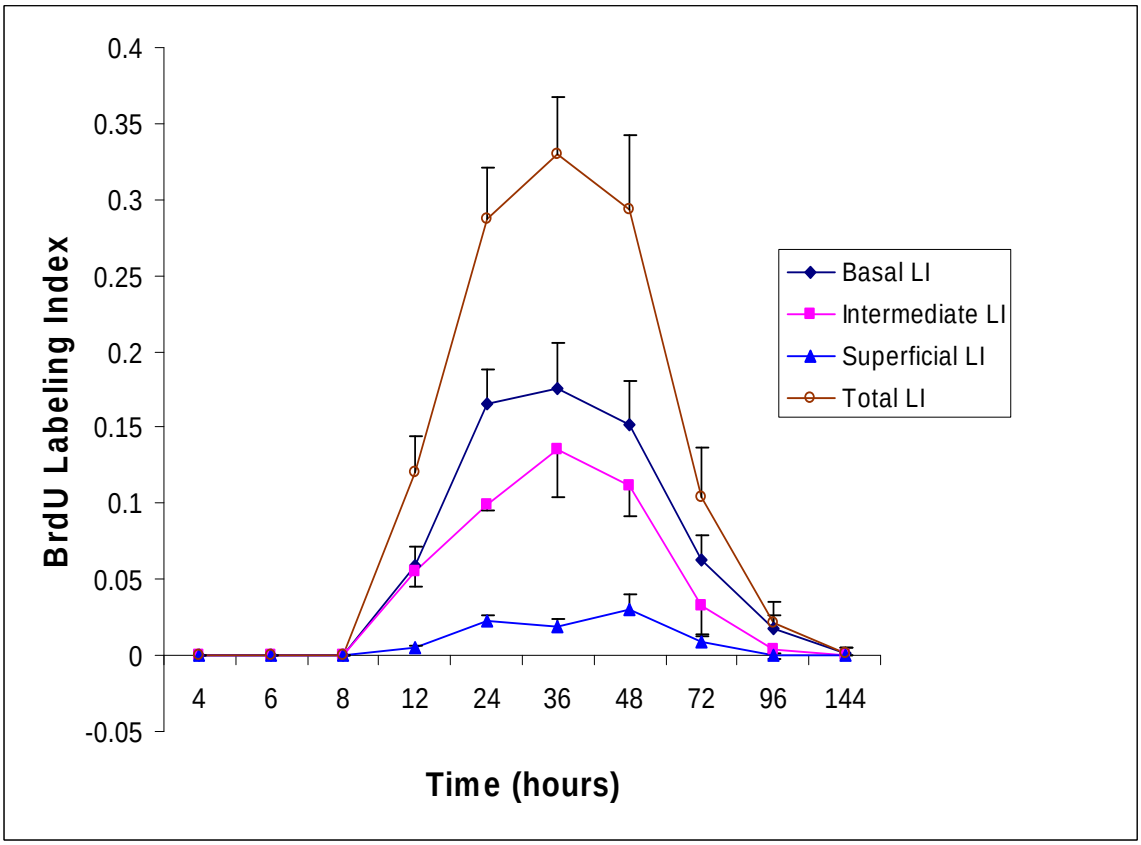
Supplementary Table

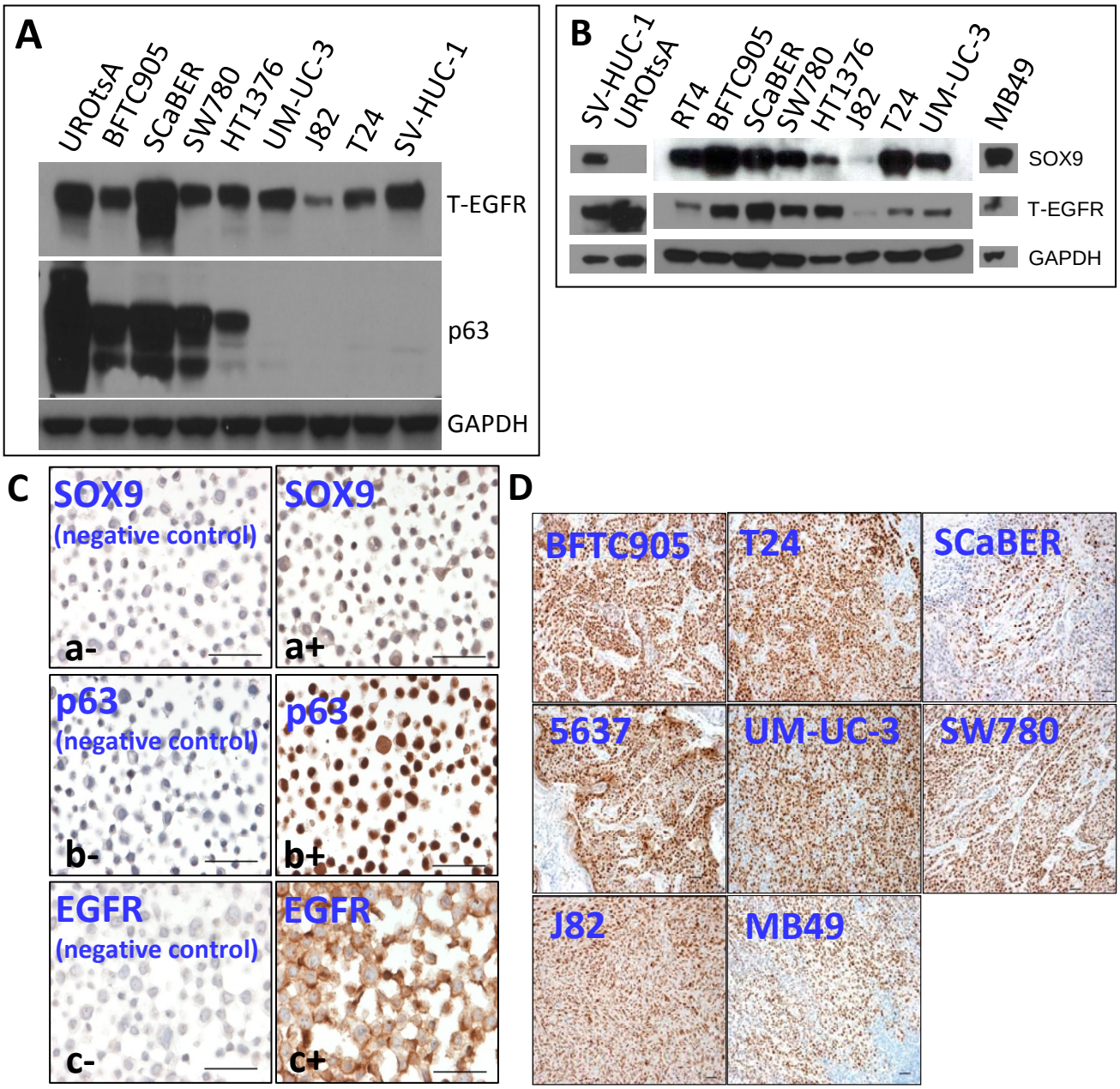
Supplementary Table 1. Real-time RT-PCR primers.

Gene name	Forward primer	Reverse primer
Egfr	GACCTTCACATCCTGCCAGT	GCATGGAGGTCAGTCCAGTT
Her2	CTCAAAACAGCTCGGAGACC	CTCATCCGGGTACTTCCAGA
Her3	CGCCAGATGACAAGCAGTTA	AGGTCATCAACTCCCAAACG
Her4	ATGCAACCACCTGTGTTCAA	GAGCCGTTTTCAAACTCTCG
Amphiregulin (Areg)	CATTATGCAGCTGCTTTGGA	GTCGTAGTCCCCTGTGGAGA
Egf	ACGCCGAAGACTTATCCAGA	CATGCTGCCTTGAAGACGTA
Hb-egf	CCCAAGCAAAGAAAGGAATG	GGCATTTGCAAGAGGGAGTA
Tgfa	AGCATGTGTCTGCCACTCTG	TGGATCAGCACACAGGTGAT
Betacellulin (Btc)	GCACAGGTACCACCCCTAGA	GCCCCAAAGTAGCCTTTCTC
Epiregulin (Ereg)	TCTGACATGGACGGCTACTG	CGCAACGTATTCTTTGCTCA
Epigen (Epgn)	CTCCTAGCACAGCACAGCAG	GCTTCAGCTCATGGTGGAAT
Neuregulin 1 (Nrg1)	CAGCAACCCAAGTTCTGTGA	TGCTGGGTTAGTCCTGCTCT
Hprt1	TATGCCGAGGATTTGGAAAA	AATCCAGCAGGTCAGCAAAG

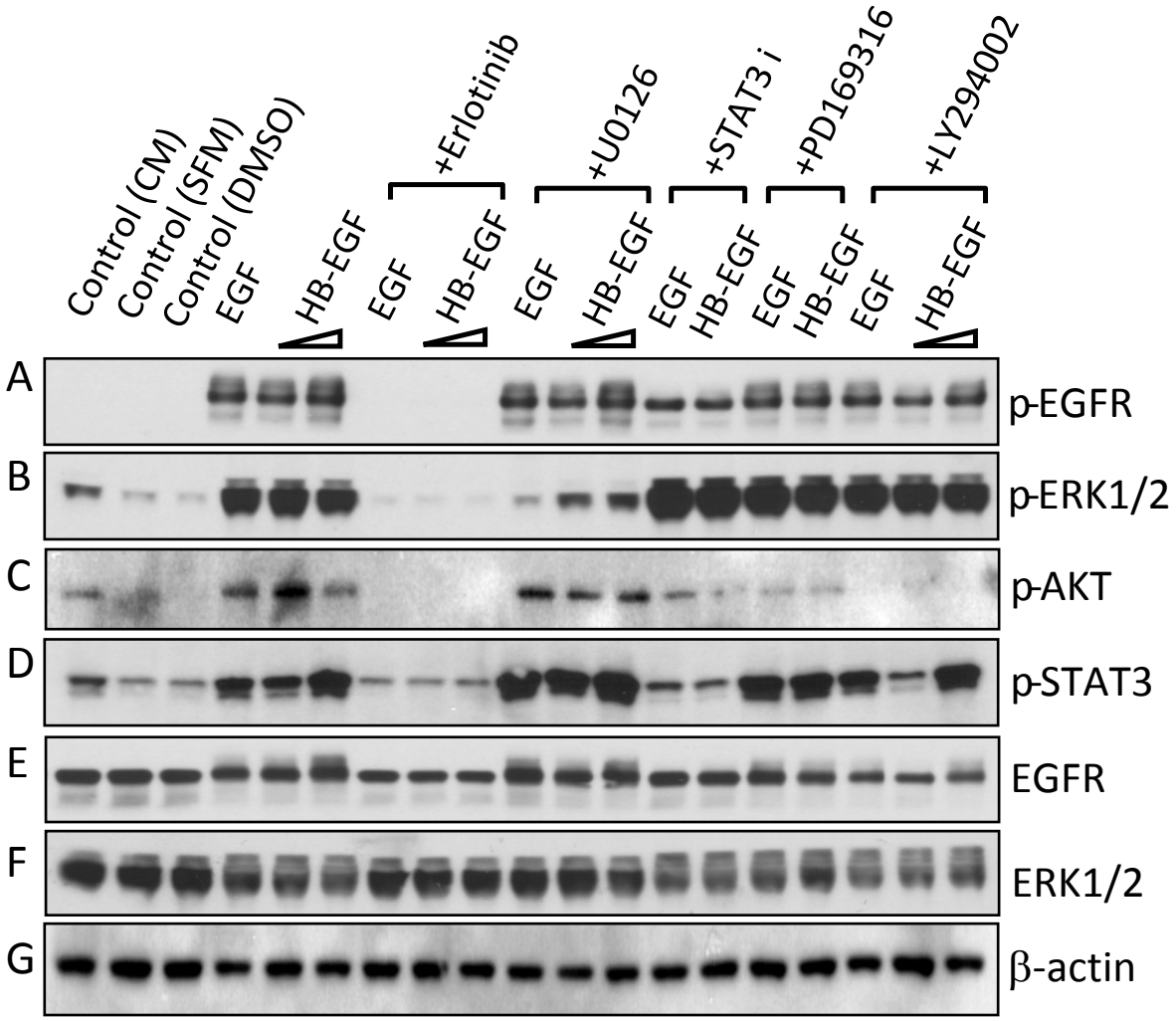
Supplementary Figures



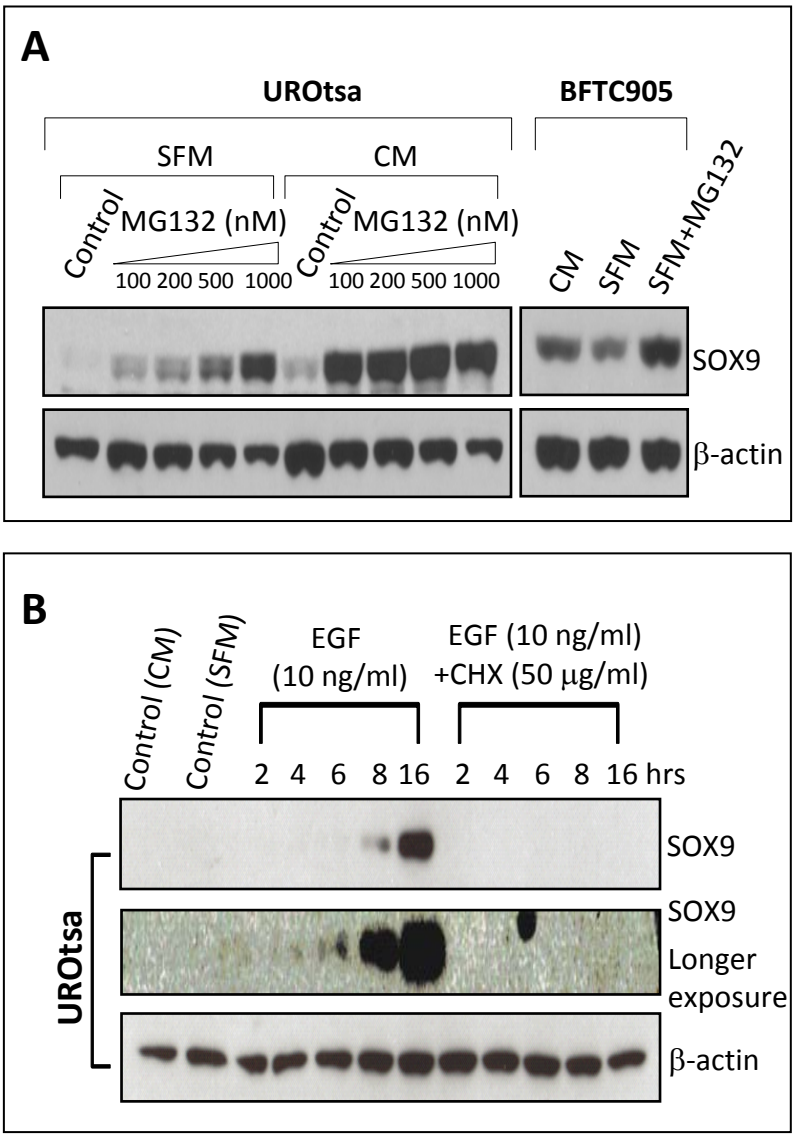
Supplementary Figure 1. Time Course of Proliferation Assay by BrdU labeling index (LI). The BrdU labeling indices for the basal cell compartment, intermediate cell compartment, superficial cell compartment, and all the three compartments were plotted. This experiment was independently repeated 3 times with 5 mice for each time point.



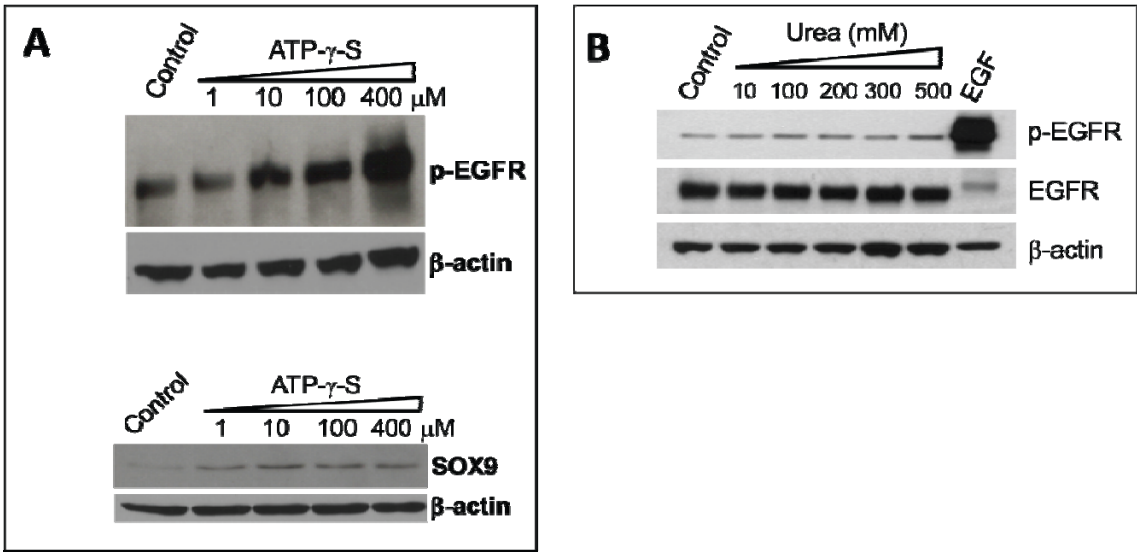
Supplementary Figure 2. Expression of EGFR, p63, and SOX9 in benign and malignant urothelial cell lines. A & B, Expression profiles of total EGFR (T-EGFR), p63, and SOX9 were examined by Western blotting in the indicated human bladder urothelial cell lines. GAPDH was used as the loading control. Note that immunoblots in panel B were performed on the same membrane, but the sample order was changed. **C**, Expression profiles of SOX9, p63, and EGFR in UROtsa by IHC staining (panel C, right, a+, b+, c+, respectively) performed on paraffin embedded sections from cell pellets. Primary antibodies were omitted for negative control (panel C, left, a-, b-, c-). p63 (nuclei stain) and EGFR (membrane stain) stained intensely, whereas SOX9 was barely detectable. **D**, Intense nuclear SOX9 staining was detected in xenografts of all UroCa cell lines tested. Photomicrographs show IHC staining for SOX9 on xenograft sections. The scale bar for all images is 50 microns.



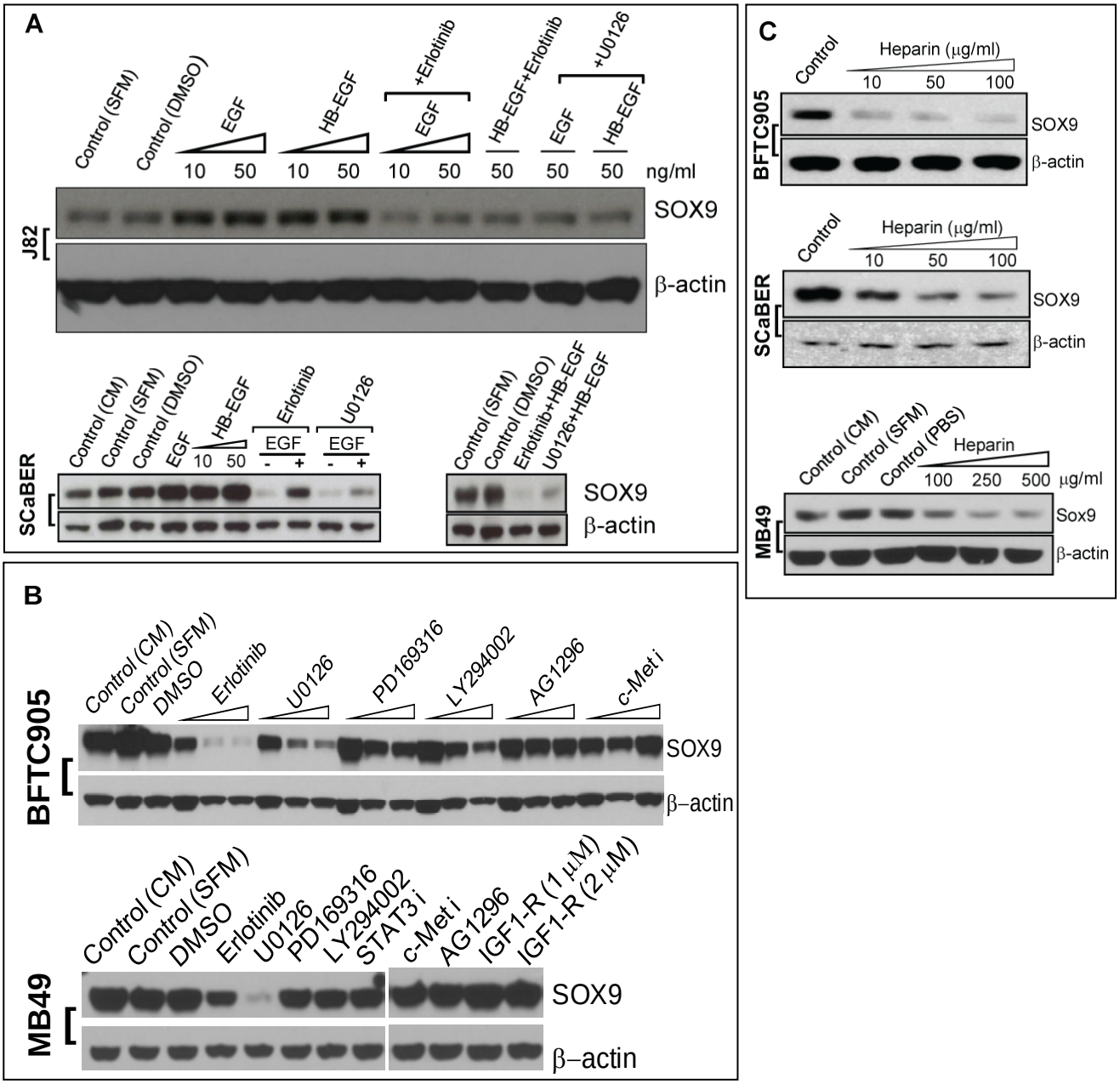
Supplementary Figure 3. Characterization of EGF- and HB-EGF-activated signaling pathways in UROtsa cells. UROtsa cells were pre-treated with the indicated inhibitor (Erlotinib, EGFR inhibitor; U0126, MEK1/2 inhibitor; STAT3 i, STAT3 inhibitor; PD169316, p38 MAPK inhibitor; LY294002, PI3K inhibitor) for 2 hrs before treatment with EGF (10 ng/ml) or HB-EGF (10 or 50 ng/ml) for 15 minutes, followed by Western blotting for phosphorylated EGFR (**A**), ERK1/2 (**B**), AKT (**C**), or STAT3 (**D**) as well as total EGFR (**E**) and total ERK1/2 (**F**). β-actin is the loading control (**G**). These results demonstrate that at the doses used, the inhibitors are effective and specific for their intended targets.



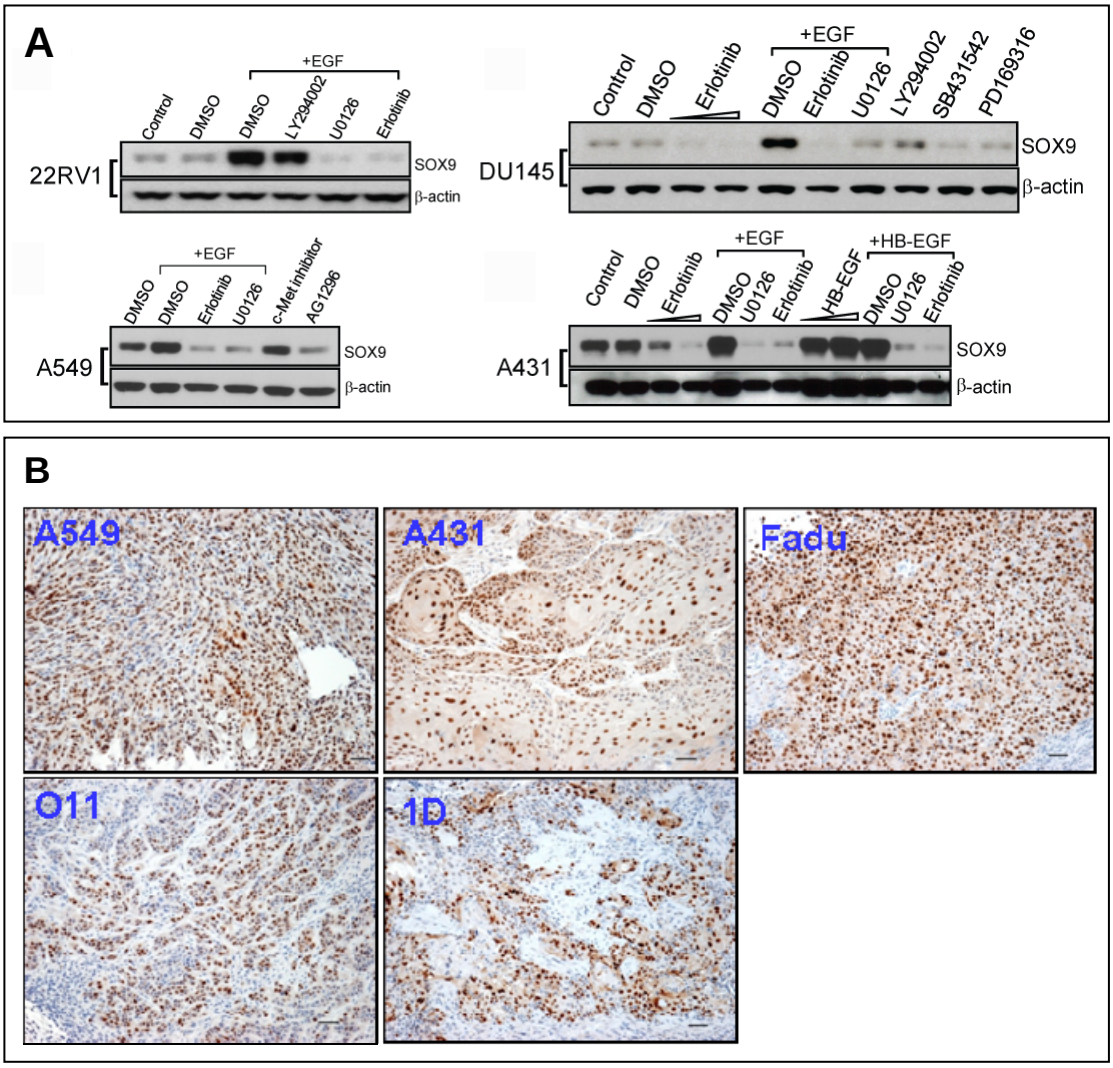
Supplementary Figure 4. Steady-state SOX9 levels are regulated by proteosomal degradation and *de novo* synthesis. A, UROtsa or BFTC-905 cells were cultured in serum-free medium (SFM) or complete medium (CM) for 1 day, then media was refreshed and the proteosome inhibitor (MG132) was added at the indicated concentrations (UROtsa) or at 1 μM (BFTC905) for 6 hrs, followed by immunoblotting for SOX9. **B,** UROtsa cells were treated with the *de novo* protein synthesis inhibitor cycloheximide (CHX) or vehicle control for 2 hours in serum-free medium before addition of EGF for the indicated duration, followed by Western blotting for SOX9 expression. These results demonstrate that proteosomal degradation maintains low SOX9 steady-state levels in benign UROtsa cells and that EGF rapidly induces *de novo* SOX9 protein synthesis.



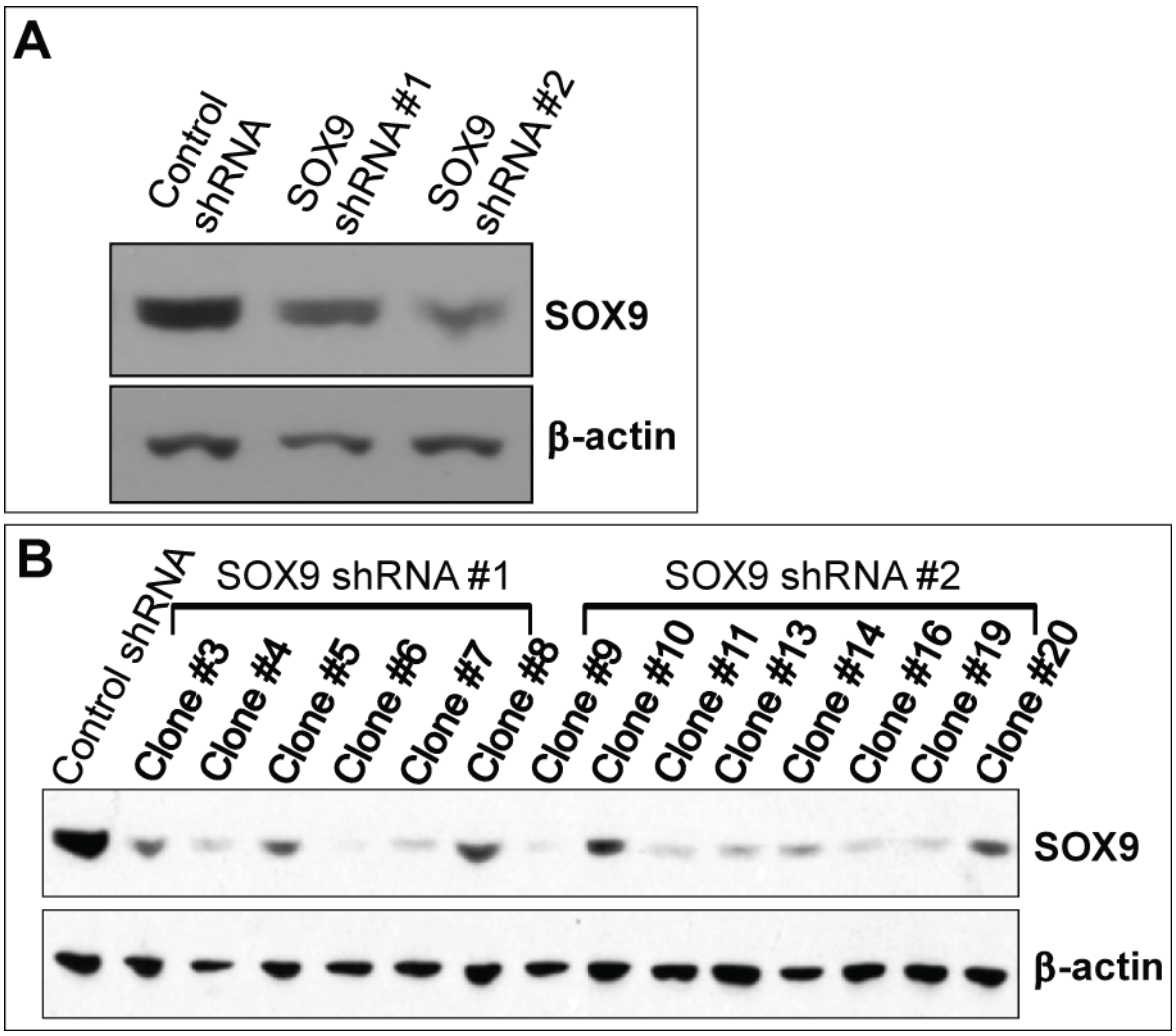
Supplementary Figure 5. Ligand-independent activation of EGFR and subsequent induction of SOX9. UROtsa cells were treated with the G protein coupled receptor ligand ATP analog, ATP-γ-S (**A**) or Urea (**B**) for 15 minutes (top panels) or 24 hrs (bottom panels), followed by Western blotting for phospho-EGFR, total EGFR, and SOX9. EGF (10 ng/ml) was used as a positive control.



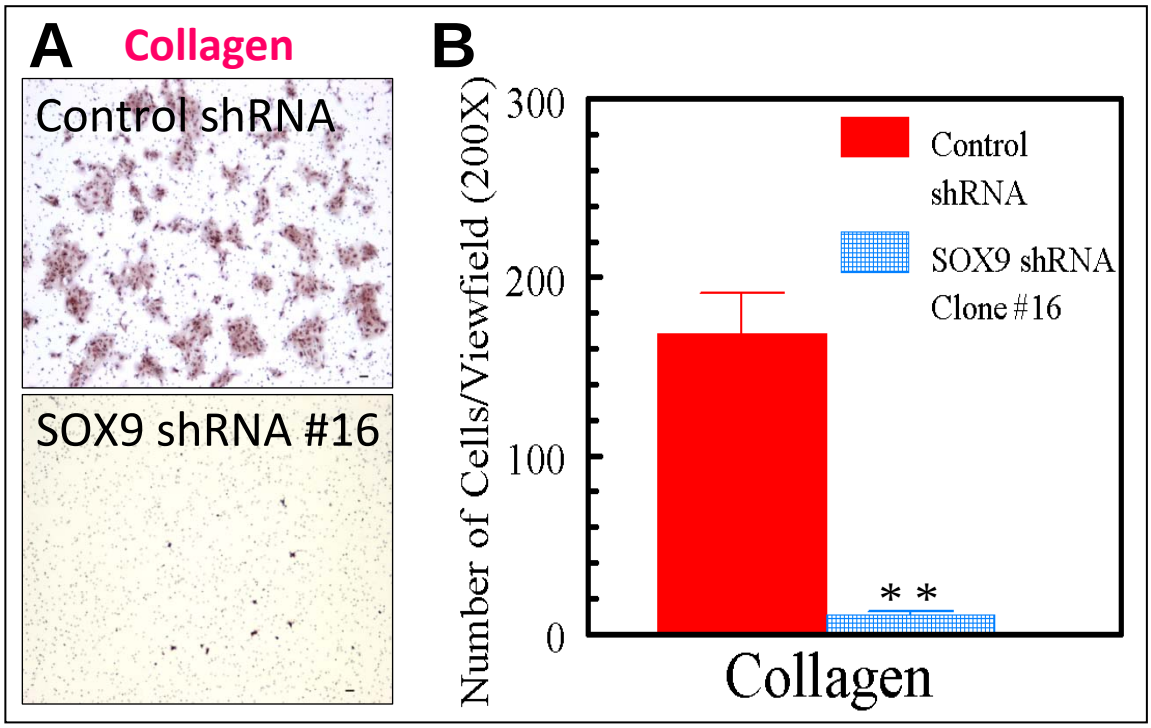
Supplementary Figure 6. Ligand-stimulated SOX9 expression in UroCa. A & **B**, Serum-starved human J82 (**A**), SCaBER (**A, C**), and BFTC905 (**B, C**), and murine MB49 (**B, C**) UroCa cells were treated with the indicated inhibitor (Erlotinib, EGFR inhibitor; U0126, MEK1/2 inhibitor; PD169316, p38 MAPK inhibitor; LY294002, PI3K inhibitor; AG1296, PDGFR inhibitor; c-Met i, c-Met inhibitor; STAT3 i, STAT3 inhibitor; IGF-1R, IGF-1R inhibitor) for 24 hrs (panel **B**) in serum-free medium (SFM), or pre-treated with the indicated inhibitors for 2 hrs (panel **A**) before co-treatment with the indicated concentration of EGF or HB-EGF for an additional 24 hrs in serum-free medium (panel **A**), or treated with heparin dissolved in PBS at the indicated dosage for 24 hrs (panel **C**) in serum-free medium, followed by Western blotting for SOX9 and β-actin. A complete medium (CM) control was also included. Note that compared with the low dose (1 μM) of each of these inhibitors, the reduced SOX9 band size seen in BFTC905 cells treated at higher doses (5 and 10 μM) of PD169316, LY294002, or c-Met inhibitor, was due to drug toxicity as evidenced by the lower expression level of β-actin.



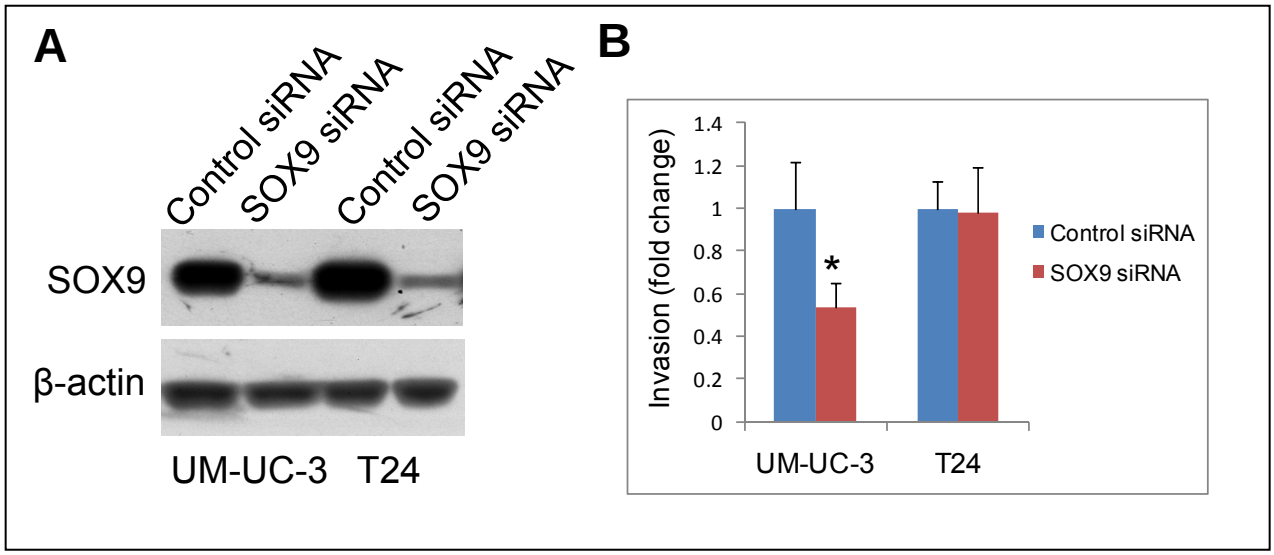
Supplementary Figure 7. EGFR-ERK1/2 dependent induction of SOX9 expression and SOX9 expression in non-urothelial cancers. A, prostate (22RV1 and DU145), lung (A549), and skin epidermoid carcinoma (A431) cells were either treated with the indicated inhibitor (Erlotinib, EGFR inhibitor at 1 or 2 μM; U0126, MEK1/2 inhibitor; LY294002, PI3K inhibitor; PD169316, p38 MAPK inhibitor, AG1296, PDGFR inhibitor; SB431542, TGFβIIR inhibitor) for 24 hrs, or pre-treated with the indicated inhibitor for 2 hrs before treatment with EGF (10 ng/ml) or HB-EGF (10 or 50 ng/ml) for 24 hrs in serum-free media. Western blots were performed for SOX9 and β-actin. **B,** Strong nuclear SOX9 staining was detected by IHC performed on histologic sections of xenografts inoculated from the indicated non-urothelial carcinoma cell lines, A549, A431 (see above), Fadu (squamous cell carcinoma of the hypopharynx), O11 (head and neck squamous cell carcinoma), and 1D (head and neck squamous cell carcinoma). The scale bar for all images is 50 microns.



Supplementary Figure 8. Selection and validation of single clones of SOX9 stable knockdown in BFTC905 cells. A, Western blot confirming SOX9 knockdown in pools of BFTC905 UroCa cells stably transfected with control or two independent SOX9 shRNA expression constructs. **B**, Western blot analysis showing different degrees of SOX9 knockdown in individual clones from pools shown in A. Clones 5 and 16 were used in the assays of growth, migration and invasion (see main text, Fig. 6, and Supplementary Fig. 9).



Supplementary Figure 9. SOX9 knockdown reduces BFTC905 cell invasion. A, Representative photomicrographs showing hematoxylin-stained cells that invaded through the collagen layer and across an 8 micron pore-size filter insert. **B,** The quantitative data shown are averaged across 3 independent experiments. The average number of invading cells were calculated from in 10 randomly viewed microscopic fields (200X magnification) per well in 3 wells for each condition in each experiment. Values are expressed as mean ± SEM. **: P<0.01. This experiment was independently performed 3 times (*n*=3).



Supplementary Figure 10. Transient SOX9 knockdown reduces UM-UC-3 cell invasion, but not T24. A, Western blots showing that SOX9 was knocked down by ON-TARGET*plus* siRNA pool in UM-UC-3 and T24 cells. **B,** The quantitative data shown are averaged across 3 independent experiments. The average number of invading cells were calculated from in 10 randomly viewed microscopic fields (200X magnification) per well in 3 wells for each condition in each experiment. Fold change was displayed. Values are expressed as mean ± SEM. *: P<0.05. This experiment was independently performed 3 times (*n*=3).

Supplementary Methods

Supplementary Method 1

Animals, mouse bladder injury model, and *in vivo* BrdU labeling C56BL6 mice (age: 6-8 weeks) were obtained from the Jackson Laboratory. Mice were maintained at 12h light-dark cycle in a pathogen-free environment with free access to water and regular feed. This protocol was approved by the animal care and use committee of the Johns Hopkins University. Mice were randomly selected for a single intraperitoneal (i.p.) injection of 250 mg/kg body weight of cyclophosphamide (CPA) or Phosphate Buffered Saline (PBS) for control in 0.2 mL volume. For *in vivo* BrdU labeling, mice (PBS control and CPA) were further administered with BrdU at a dosage of 1 mL concentrated BrdU labeling reagent (Zymed, South San Francisco, CA) per 100 g body weight by a single intraperitoneal injection two hours before sacrifice. Mice were euthanized and bladders were harvested at the indicated time points (hours after CPA injection). The whole bladders were fixed in 10% neutral-buffered formalin for histology and BrdU labeling index using the BrdU staining kit per the manufacturer's instructions (Zymed, South San Francisco, CA). The BrdU labeling indices were calculated as the ratio between the number of the BrdU-positive cells and total number of cells in the basal cell compartment, intermediate cell compartment, or superficial cell compartment as well as in all three compartments in 10 random high power fields (40X) in each section.

Supplementary Method 2

Details for RHS4531-NM_000346

Product Page	(click to open) - (see related products)
Catalog Number	RHS4531
Clone Id	V2LHS_11387, **V2LHS_92504**, **V2LHS_92503**
Cluster	Hs.707993
Description	Human GIPZ lentiviral shRNAmir target gene set
Accessions	NM_000346, BC018276, Z46629
Species	Homo sapiens
Location	172_0058-A-4
Vector Name	pGIPZ
Vector Type	Lentiviral
Antibiotic Information	Zeocin (Concentration: 25 µg/ml, Resistant Range: 25-25 µg/ml); Ampicillin (Concentration: 100 µg/ml, Resistant Range: 100-100 µg/ml)
Additional Information	Lentiviral vector

Details for RHS4531-NM_000346

TGCTGTTGACAGTGAGCGCGCAGTTAACCTTCAAGACATTTAGTGAAGCCACAGATGTA
AATGTCTTGAAGGTTAACTGCTTGCCTACTGCCTCGGA

Color Codes: mir-30 context sense loop antisense

Hairpin sequence for V2LHS_92503

TGCTGTTGACAGTGAGCGACCTGTTTGGACTTTGTAATTATAGTGAAGCCACAGATGTA
TAATTACAAAGTCCAAACAGGCTGCCTACTGCCTCGGA

Color Codes: mir-30 context sense loop antisense

Hairpin sequence for V2LHS_92504

TGCTGTTGACAGTGAGCGCGCTTGTTCACTGCAGTCTTAATAGTGAAGCCACAGATGTA
TTAAGACTGCAGTGAACAAGCATGCCTACTGCCTCGGA

Color Codes: mir-30 context sense loop antisense

Reference Sequence for: NM_000346

>Homo sapiens SRY (sex determining region Y)-box 9 (SOX9), mRNA.

ggagagccgaaagcggagctcgaaactgactggaaacttcagtggcgcggagactcgcca

gtttcaaccccggaaacttttctttgcaggaggagaagagaaggggtgcaagcgccccca

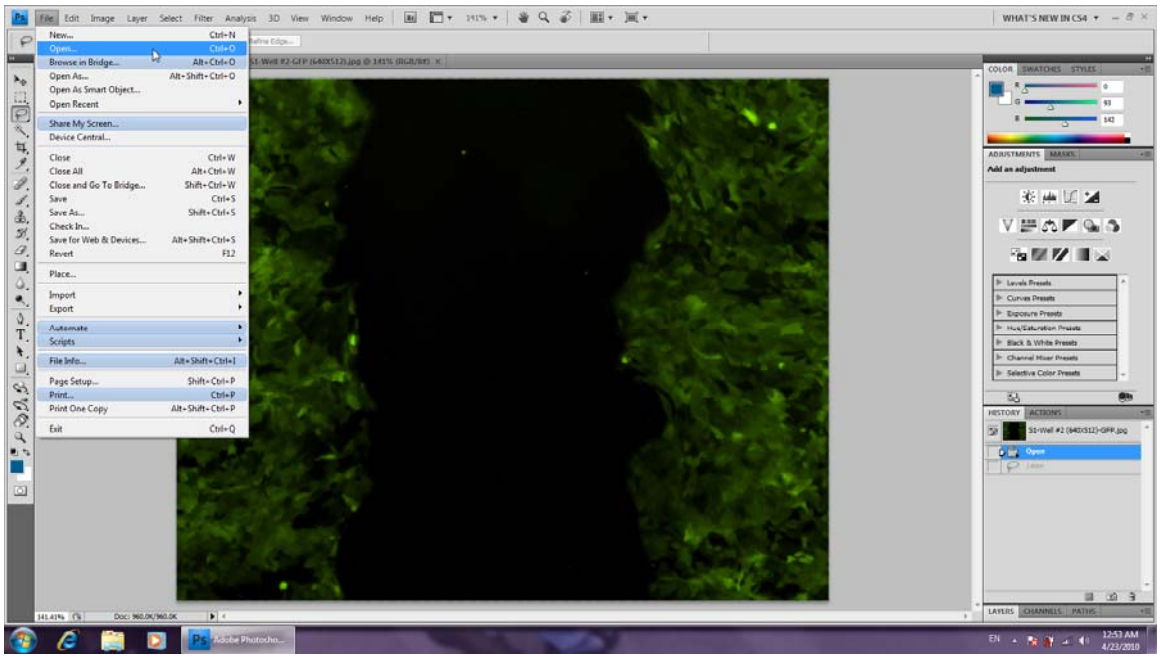
siRNA transient transfection and invasion assay ON-TARGET*plus* SMARTpool control siRNA and human SOX9 siRNA were purchased from Dharmacon (Lafayette, CO) and cells were transfected with RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The SOX9 siRNA pool contains 4 siRNAs targeting different regions of SOX9. Briefly, T24 and UM-UC3 cells were plated at a density of 1×10^5 cells /well in 6-well plates. The following day, a stock solution containing 2 μl (20 μM) of siRNA and 4 μl of transfection reagent per 500 μl of OptiMEM media (Gibco, Carlsbad, CA) was prepared and incubated at room temperature for 20 min. Medium was aspirated from each well and replaced with 1.5 ml of antibiotic-free culture medium (DMEM/10% FBS) plus 500 μl of transfection mixture. Following an overnight incubation, the transfection mixture was replaced with complete culture media. 48 hrs after transfection, cells were trypsinized and counted. Cell migration assay were conducted according to the protocol described in the Materials and Methods.

Supplementary Method 3

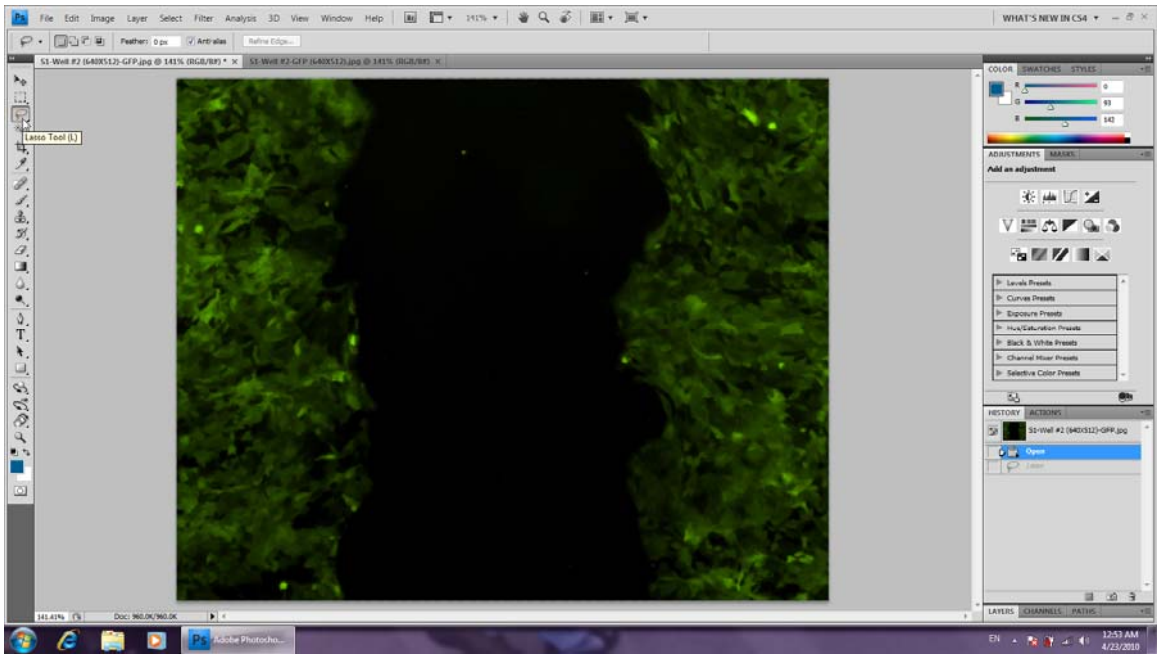
Quantification of Wound Closure Using the Analysis Function of Photoshop CS4

Step 1.

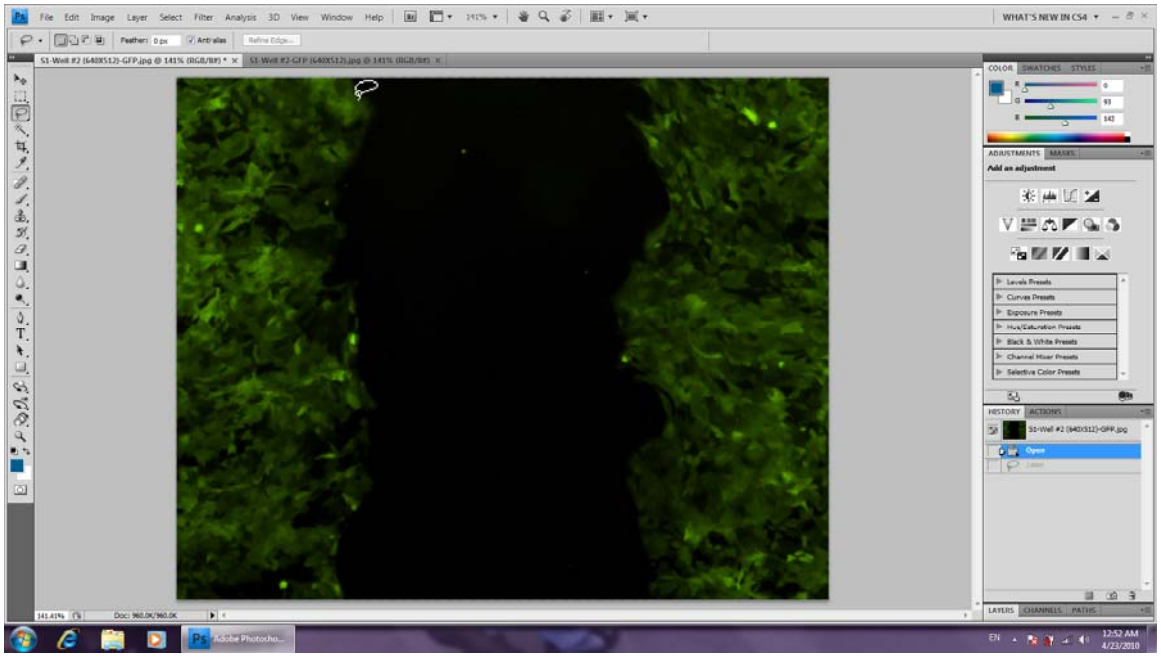
1. Open the file of a given time point, such as time 0

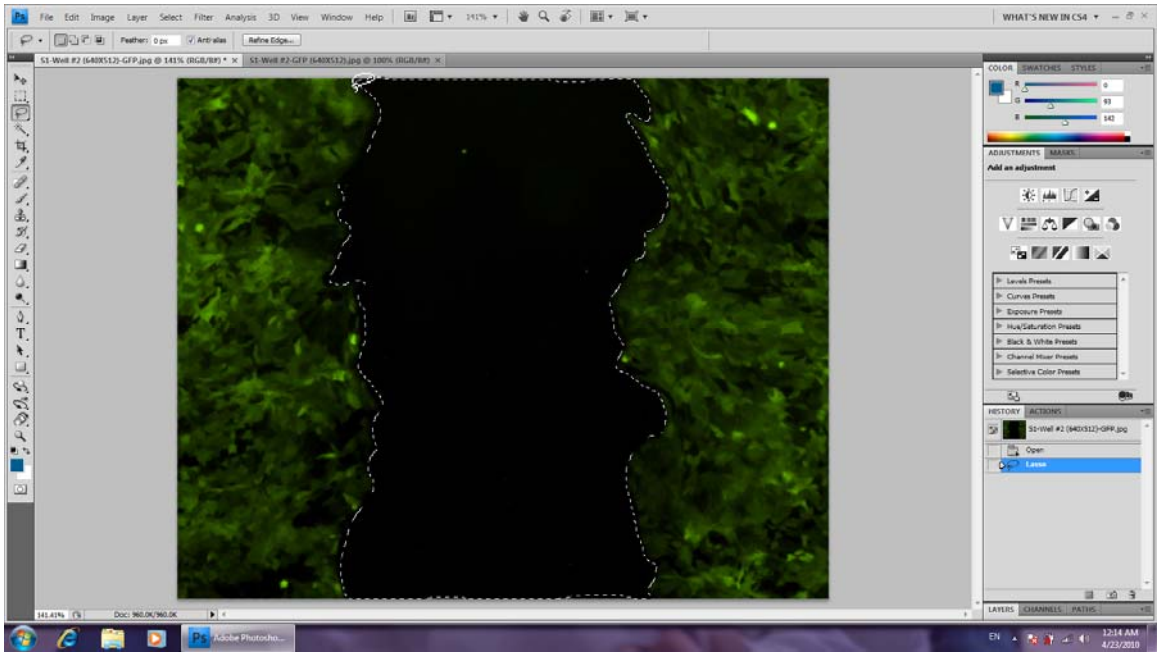


2. Choose the Lasso Tool

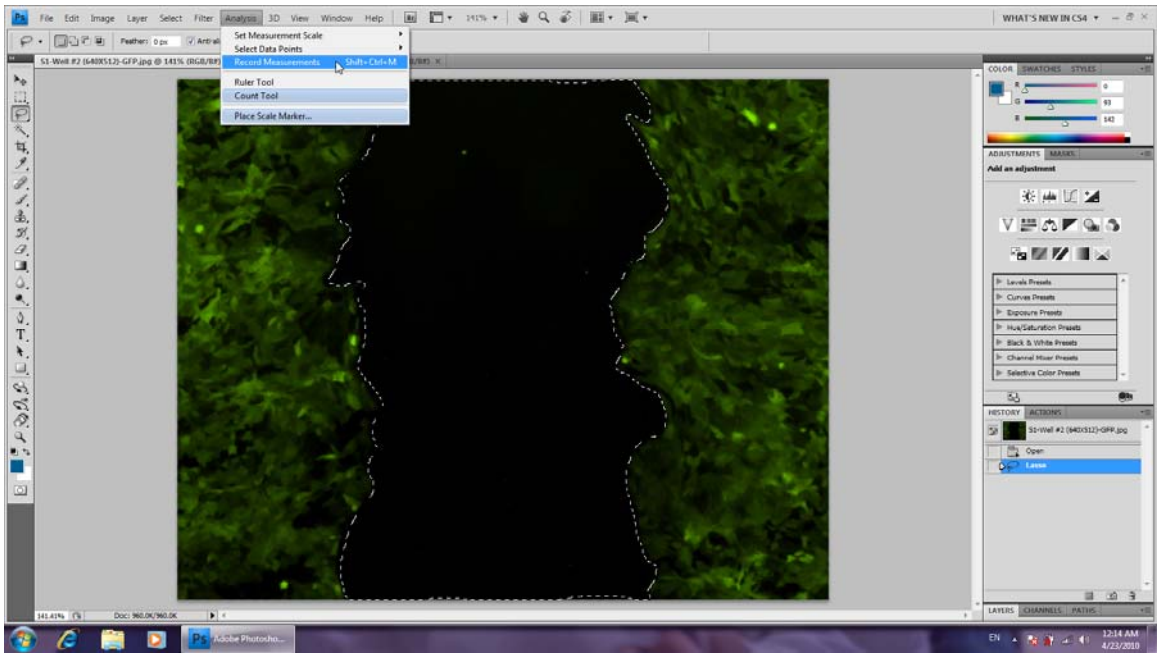


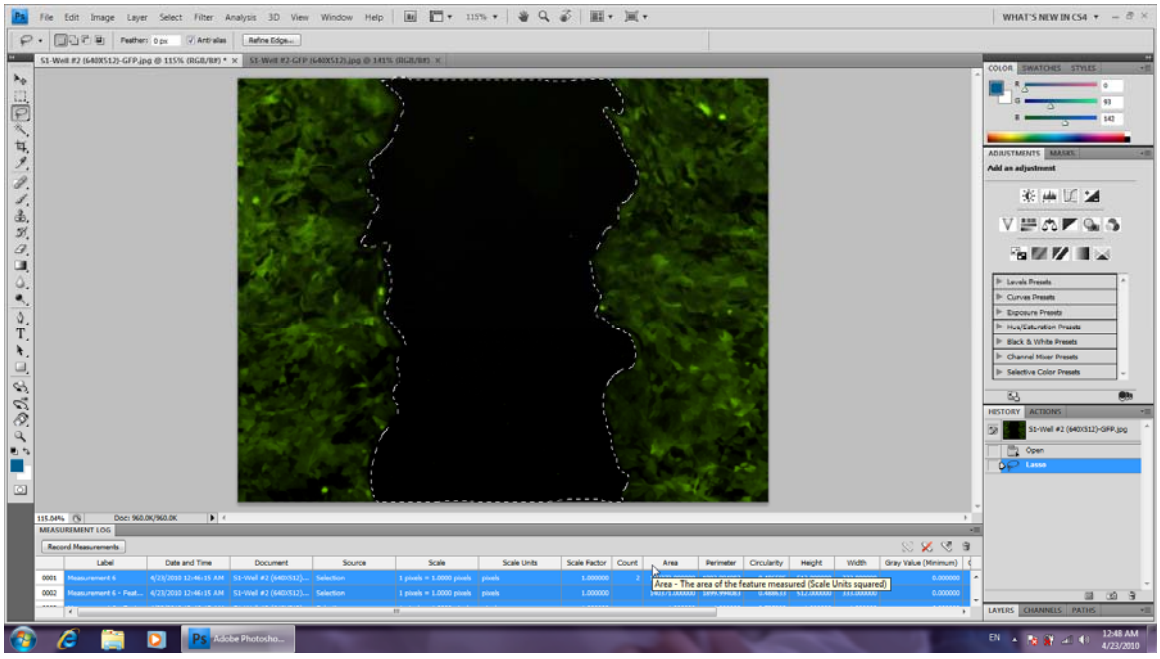
3. Trace the wound edges using the lasso tool

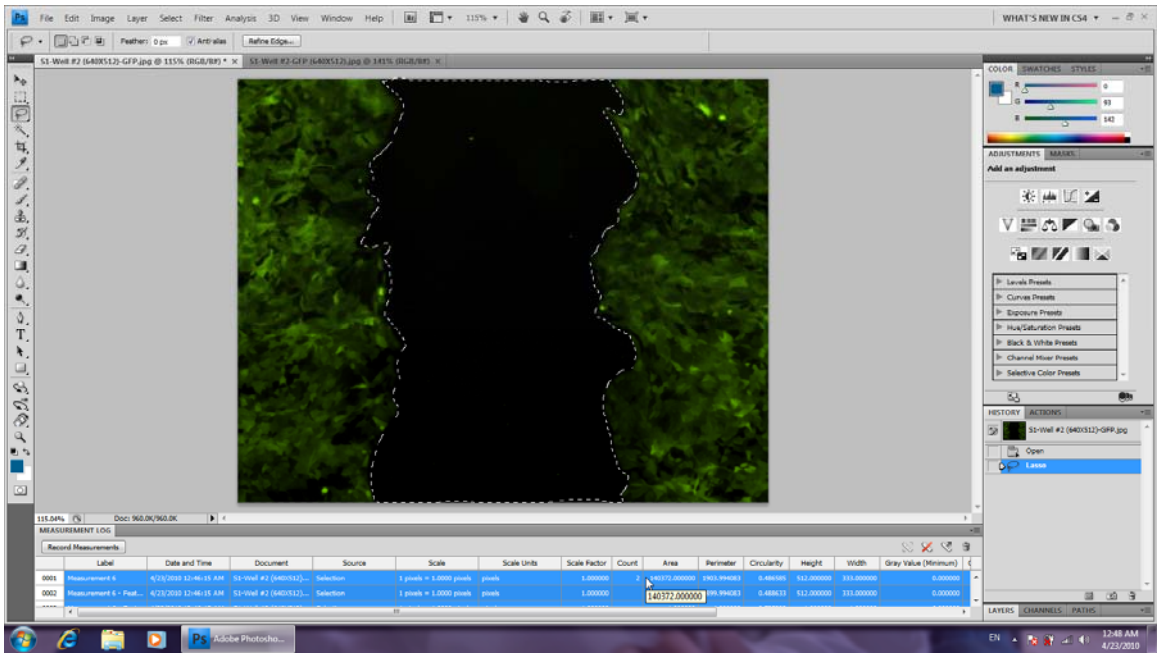




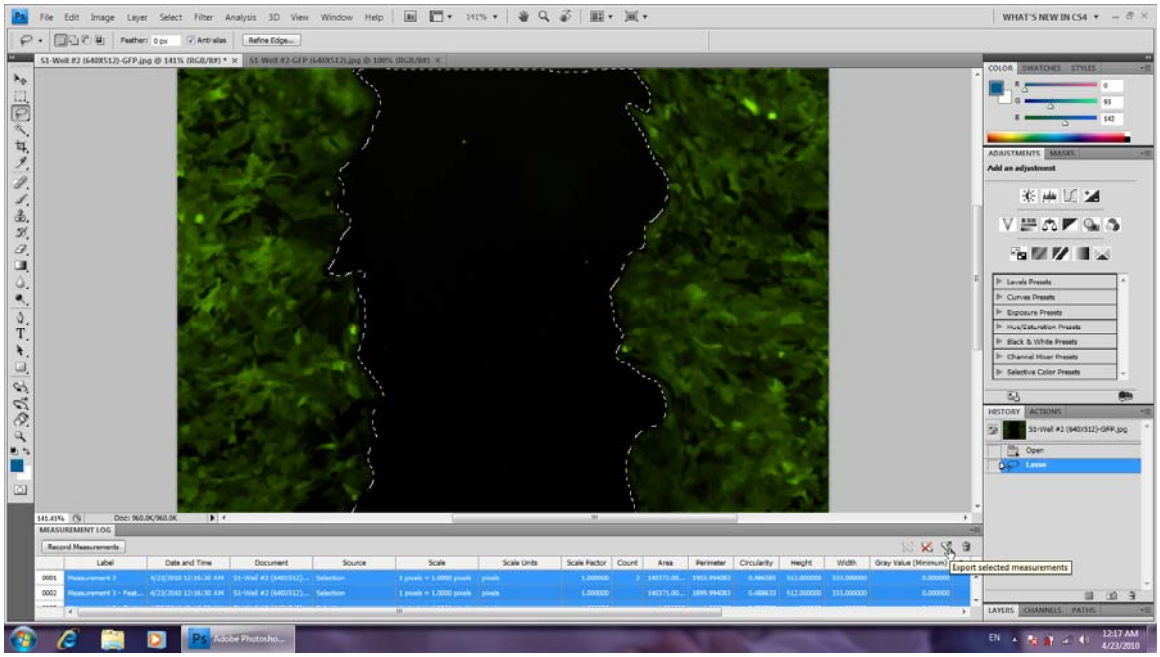
4. Record measurements of the wound area using Photoshop CS4's Analysis function in the Analysis Tab.





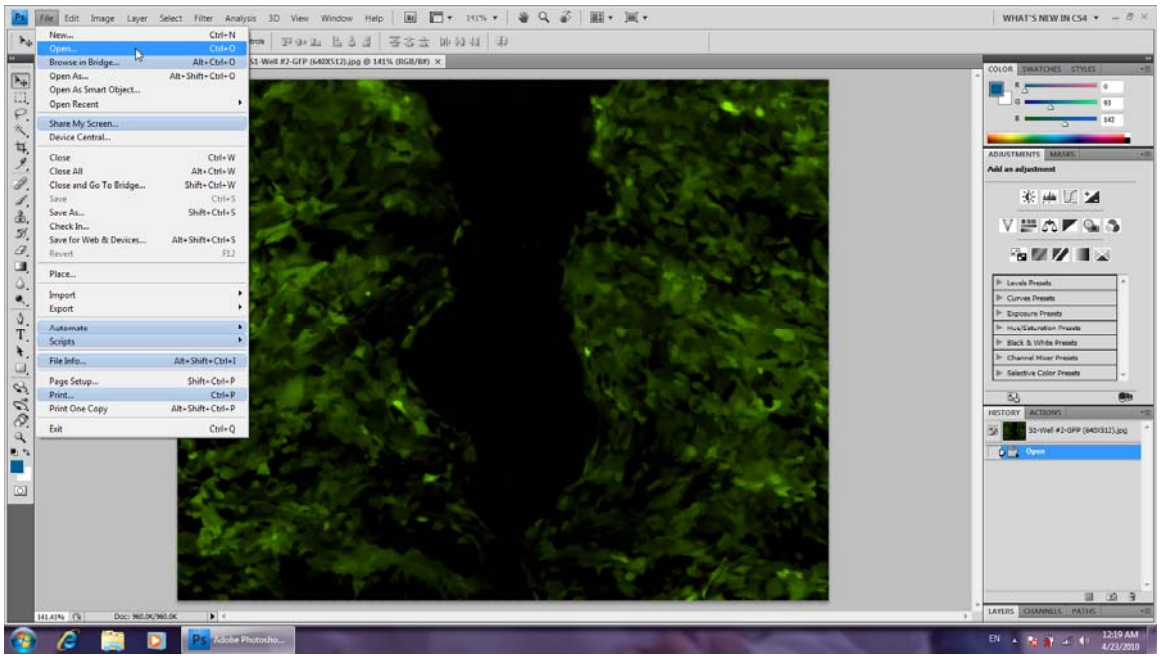


5. Export the measurement to Excel

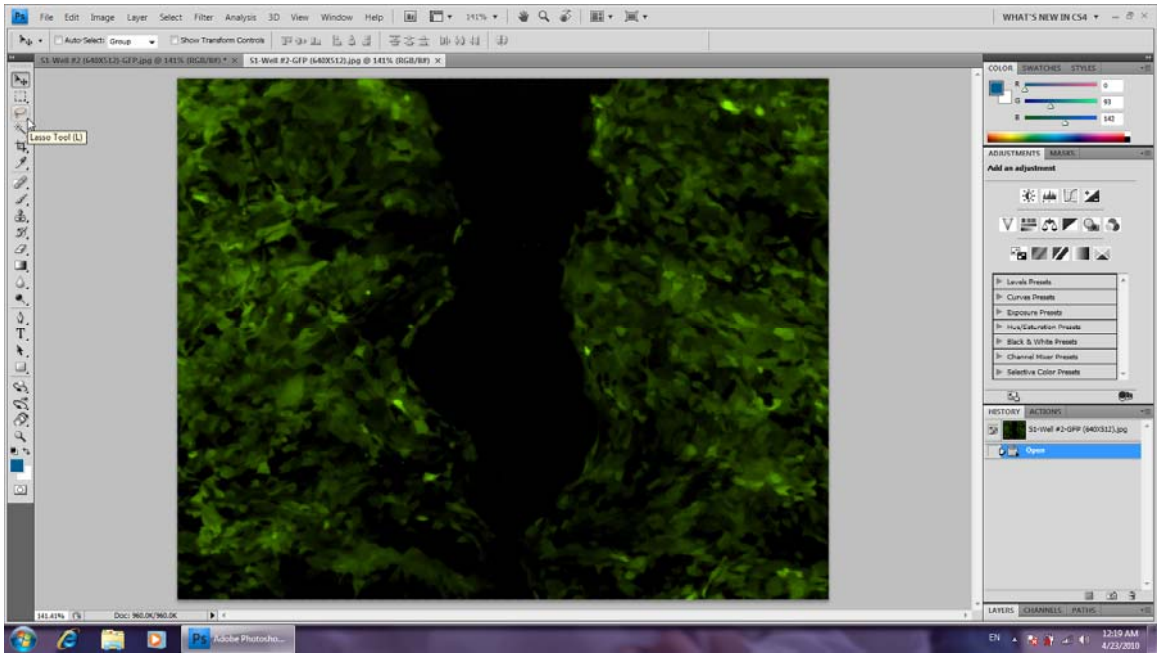


Step 2. Open the file of a later time point, such as 24 hours, then following all the steps in the Step 1, and finally calculate the percentage of wound closure between these two time points.

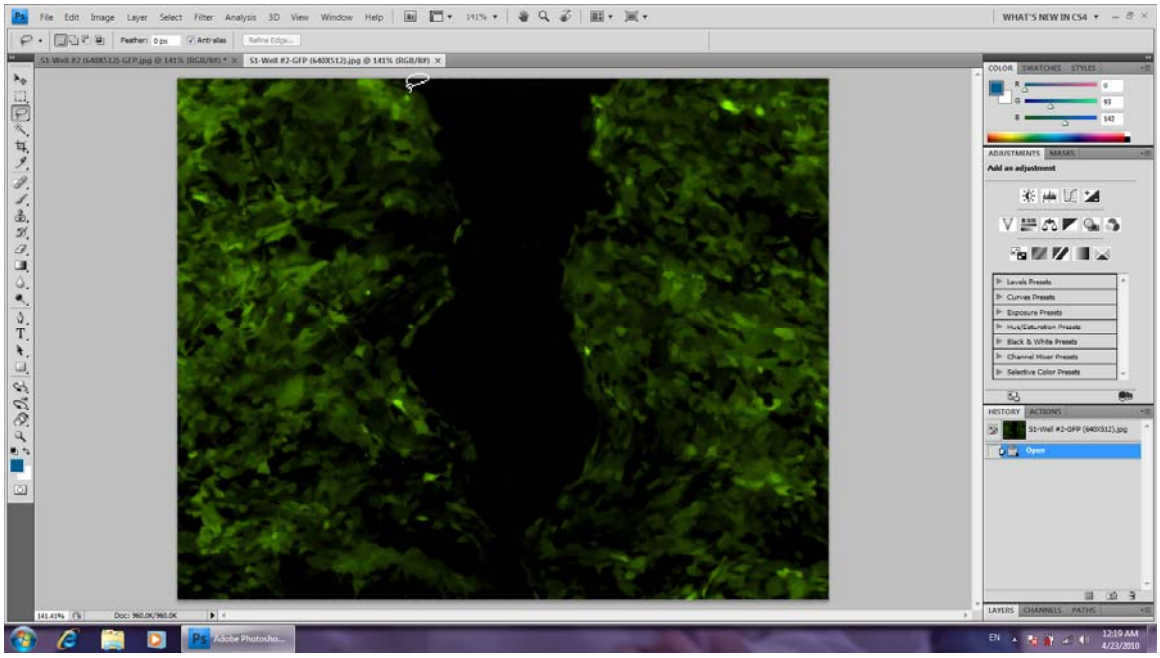
1. Open the file of a later time point, such as 24 hours

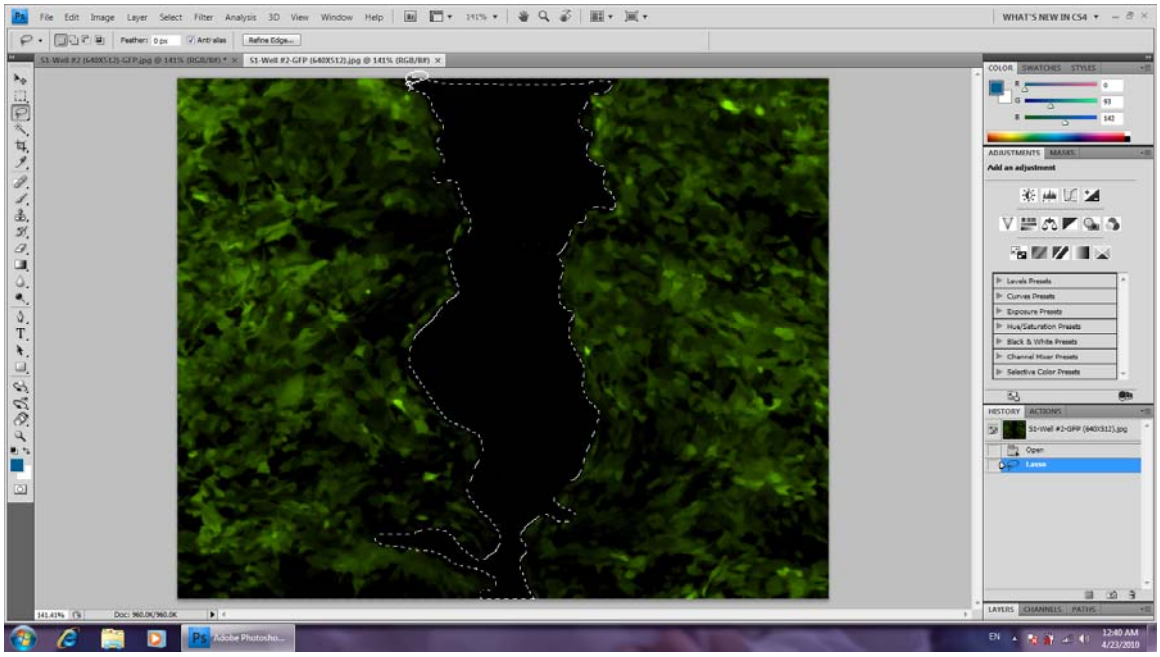


2. Choose the Lasso Tool

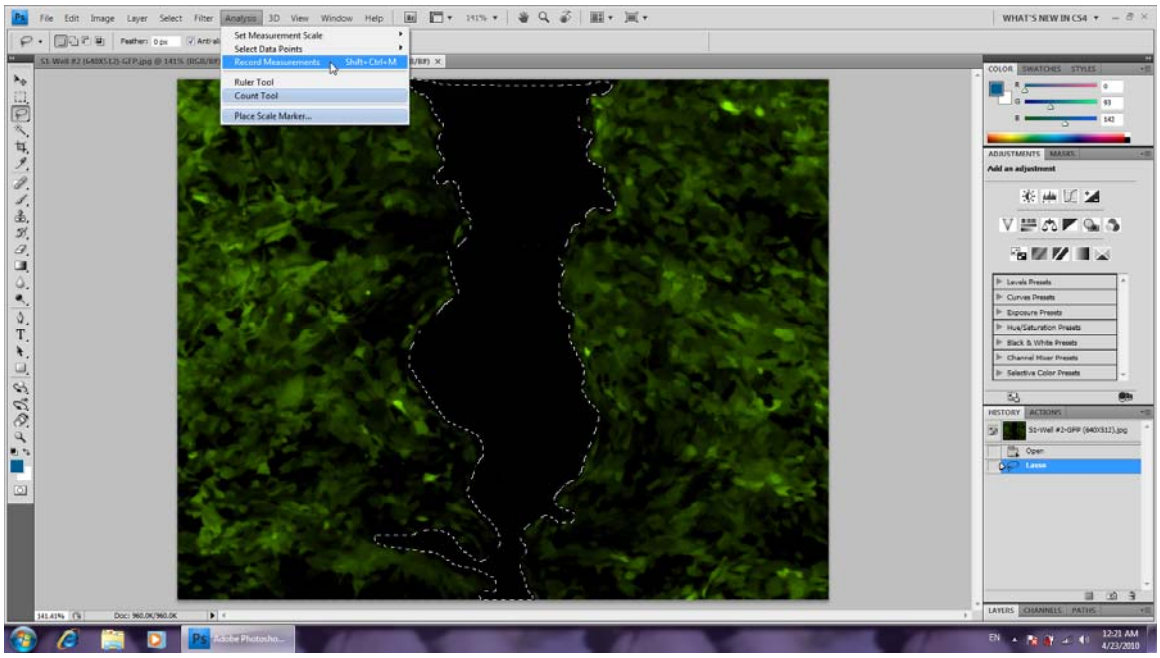


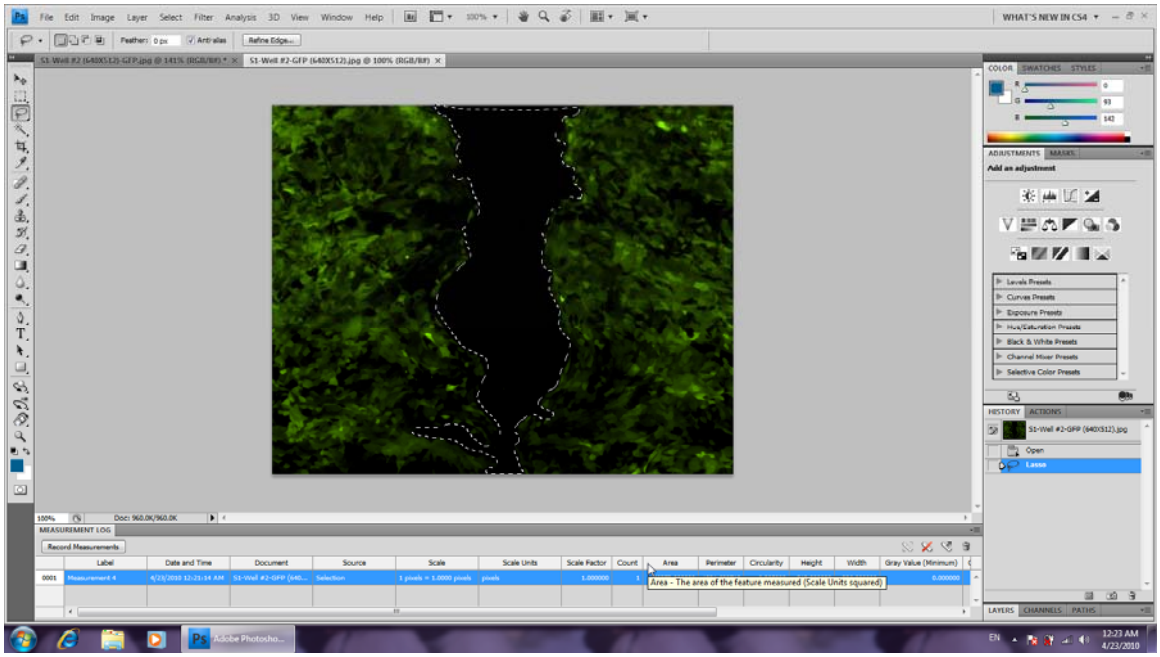
3. Use the Lasso Tool to trace the wound edges

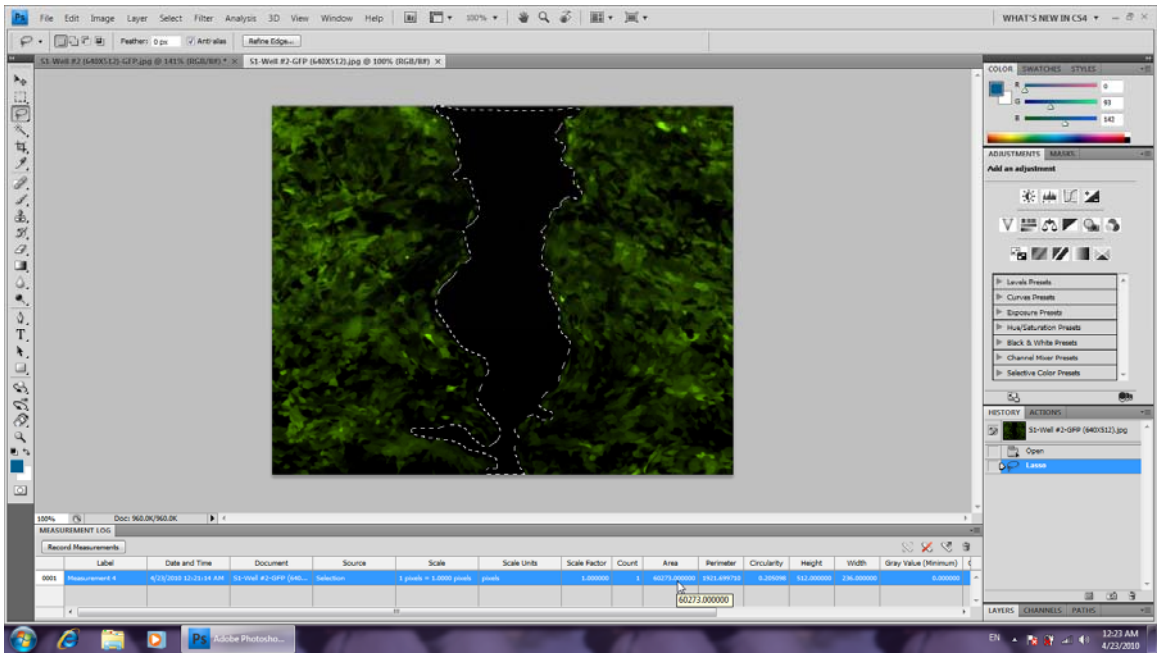




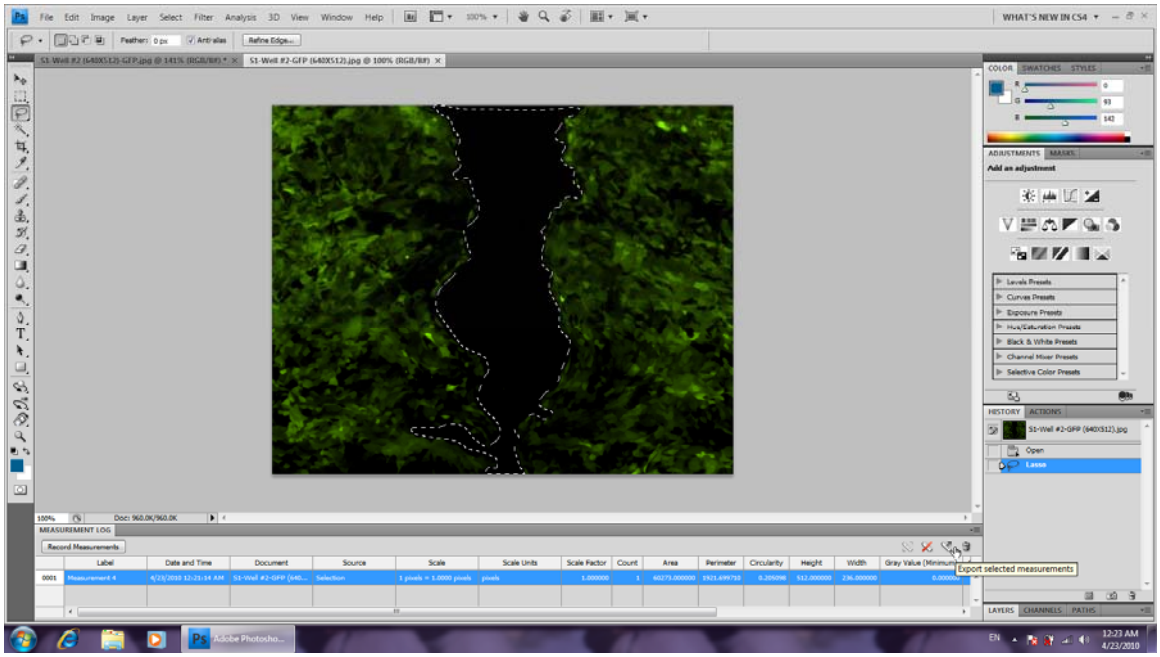
4. Record Measurements using Photoshop CS4's analysis function in the Analysis Tab.







5. Export the measurements to Excel



6. Follow all the steps in the Step 1 and Step 2, then get all measurements for other time points, such as 48 hours and 72 hours. Export all measurements to Excel to finish further analysis, such as percentage of wound closure between two points.

7. Using the same method to calculate percentages of wound closures in additional repeated experiments, followed by the calculation of mean percentages of wound closures between two points, standard errors of the means, and statistical analysis.