Supporting Information

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SI Materials and Methods

Vectors. *Lepr*-luciferase promoter reporter constructs were generated by PCR using Pfu polymerase and C57BL/6 mouse genomic DNA as a template. Primers containing upstream KpnI and downstream XhoI restriction sites were used to generate *Lepr* promoter fragments, with the exception of the −60/+395 and −60/+535 constructs, which contain upstream BglII and downstream HindIII restriction sites. Restriction-digested PCR products were subcloned into pGL3-Basic vector (Promega). Point mutants were introduced into the −60/+535 reporter by the Pfu/DpnI method using the in vitro mutagenesis kit (Invitrogen) according to the manufacturer's guidelines. All clones were confirmed by sequencing on both strands.

The Oct4 (−5068)-Luc reporter vector (1) was obtained from Addgene (plasmid 17221). The Nanog (−4828/+190)-Luc reporter containing 4.8 kb of the 5′ flanking region of the mouse *Nanog* gene was obtained from Da Yong Wu, Shanghai Institutes for Biological Sciences (Shanghai, China). The *Sox2* (3.3-kb)-luciferase reporter vector (2, 3) was generated by PCR using Pfu polymerase and subcloned into the pGL3-Basic vector. Lentiviral-based shRNA expression vectors were obtained from Open Biosystems. The following shRNA vectors were used: V3LHS_392592 (*OCT4* #1), V3LHS_311241 (*OCT4*#2), V2LHS_153339 (*SOX2*), and RHS4346 for the nontargeting, scrambled control (sh-*Scr*).

Cells and Reagents. Liver tumors were removed surgically before euthanasia from NS5a-Tg mice (4) maintained for 12 mo on a Lieber–Decarli alcohol diet (3.5% alcohol). Tumor-initiating stem cells (TISCs) were isolated using the anti-prominin1 (CD133) MACS affinity column (Miltenyi Biotech) following mechanical dissociation of liver tumors in sterile PBS and digestion with 1 mg/mL collagenase/dispase solution (Roche) for 45 min at 37 °C. The unbound column flow-through fraction ($CD133^-$) was also recovered for propagation. Cells were maintained in TISC culture medium [DMEM-F12, 10% FBS, 100 nM dexamethasone, 1× nucleosides (Sigma), and 20 nM mouse EGF].

E14 mouse embryonic stem cells harboring a chromosomal Oct4-GFP reporter were plated in 60-mm gelatin-coated dishes in ES medium (DMEM, 15% FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μg/mL each penicillin and streptomycin) supplemented with 0.1 mM β-mercaptoethanol and either leukemia inhibitory factor (1,000 units/mL) or recombinant mouse leptin (200 ng/mL) (R&D Systems). Immortalized PIL-4 hepatoblasts were generously provided by Aleksandra Filipovska (University of Western Australia, Crawley, Australia). Rat iPS2 cells were a generous gift of Qilong Ying (University of Southern California Keck School of Medicine, Los Angeles, CA).

The following commercial antibodies were used: Stat3 (Santa Cruz Biotechnology; SC-482×), OCT4 (Santa Cruz Biotechnology; SC-8682×), SOX2 (Santa Cruz Biotechnology; SC-20088×), OB receptor (OB-R) (Santa Cruz Biotechnology; SC-1835 and SC-8391), phospho-STAT3 (p-Y705) (Abcam; 76315), CD133 (Abcam; ab27699), Ki67 (Abcam; 15580), Annexin V (Abcam; 14196), and actin (Sigma; A5316).

Reporter Assays. Early passage liver TISCs (fewer than five passages in culture) or control PIL-4 hepatoblasts cultured in six-well plates were cotransfected with 2 μg luciferase reporter and 100 ng *Renilla* luciferase expression vector to control for transfection efficiency. Forty-eight hours after transfection, cells were lysed in 1× passive lysis buffer, and luciferase activity was measured using the Dual-Glo Luciferase System (Promega) using a Lumat LB9501 luminometer (Berthold). The data shown represent the mean ± SD from at least three independent biological replicates.

Human Tissue Specimens. Flash-frozen tissues and sections from formalin-fixed and paraffin-embedded human hepatocellular carcinoma (HCC) and matched normal liver tissue surgical specimens were obtained from the University of Minnesota Liver Tissue Cell Distribution System. In all, nine sets of HCC tumor and matched normal tissue samples (from five men and four women) were obtained from patients (49–63 y of age) with premetastatic liver tumors of varying grades. The normal control tissues were obtained from the margins of the surgical specimens. All tissues were collected with informed patient consent that was granted before surgery in accordance with a protocol approved by the Institutional Review Board at the University of Minnesota, Minneapolis.

Immunohistochemistry. Mouse tumors were surgically resected upon necropsy, embedded in OCT compound (Sakura Finetek), and stored at −80 °C until processing. Five-micrometer sections were prepared using a cryostat refrigerated at −20 °C.

Paraformaldeyde-fixed, paraffin-embedded human tissue sections were rehydrated by immersion in xylene (3 × 5 min) and 100% ethanol (2 × 5 min) followed by a series of hydrated ethanol baths and PBS. To enhance antigen recovery, rehydrated sections were immersed in 10 mM sodium citrate (pH 6.0) at 90 °C for 15 min, followed by two washes in PBS. All tissue sections were blocked in PBS-2% BSA and probed with primary antibodies at a dilution of 1:400 in PBS, followed by washes (3 × 5 min) in PBS and incubation with anti-rabbit Alexa 546 and anti-mouse Alexa 488 (Molecular Probes) diluted to 1:500 in PBS. Sections were washed in PBS (4 × 5 min) and subsequently mounted in mounting medium supplemented with DAPI (Vector Laboratories).

Cell Lysis and Immunoblotting. Cultured TISCs or $CD133^-$ controls grown in 10-cm plates were rinsed with PBS, gently scraped from the plate, and lysed in cold RIPA buffer [20 mM Tris (pH 7.7), 150 mM NaCl, 1% triton X-100, 1% sodium deoxycholate, 0.05% SDS, 1 mM $NaVO_3$, 10 mM NaF] containing complete protease inhibitor mixture (Roche) for 1 h on ice, followed by centrifugation at 14,000 × *g* for 5 min and recovery of supernatant. Human tissue or mouse tumors recovered from anesthetized mice were prepared by dounce homogenization in cold RIPA buffer before centrifugation.

Protein concentration was determined using the D*c* Protein Assay Kit (Bio-Rad), and the supernatant was mixed with 6× Laemmli sample buffer before resolving by 8% SDS/PAGE and immunoblotting.

Quantitative Real-Time PCR. Total RNA was prepared with TRIzol (Invitrogen) and purified using the RNeasy mini kit (QIAGEN) with DNase I treatment. First-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed with 2× Sybr Green Master Mix (Applied Biosystems). All reactions were run on an ABI 7300 HT Real-Time PCR instrument with a 5-min hot start at 95 °C followed by 40 cycles of a three-step thermocycling program: 15 s denaturing at 94 °C, 15 s annealing at 55 °C, and a 20-s extension at 70 °C. For quantitative reverse-transcription PCR (qRT-PCR), reactions were first incubated at 48 °C for 40 min before PCR cycling. Gene expression was determined relative to GAPDH

control via the ΔCt method. The following primers were used for quantitative real-time PCR:

For OB-R (long-form) (*M. musculus*), forward (TTTGTAGAAAATAATTTGAG) and reverse (CTTGGATGAGATTACACAGT);
For IL-6R (*M. musculus*), forward (TGGCAGCTGGCAGGGCACCAC) and reverse (ACCTGTGCAGAGCTCTTGTGGGTT);
For OCT4 (*M. musculus*), forward (GGCGAGGCCTTTCCCTCTGT) and reverse (CTCAGTAAAAGAATTTAACC);
For SOX2 (*M. musculus*), forward (TGCGAACTGGAGAAGGGGAGAG) and reverse (CGCAGCTGTCGTTTCGCTGCGG);
For GAPDH (*M. musculus*), forward (TGTGTCCGTCGTGGATCTGA) and reverse (AAGTTGCAGGAGACAACCTGGTC).

Electrophoretic Mobility Shift Assay. A 5′-biotinylated nucleotide (5′-CCGAGGAATCGTTCTGCAAATCCAGGTATGTTGAGA-3′) and its 5′-biotinylated reverse complement corresponding to the candidate OCT4/SOX2 binding site in the *Lepr* gene were mixed at 50 μM, incubated at 95 °C for 5 min, and gradually cooled to room temperature before the assay. Electrophoretic mobility shift assay was performed in a 10-μL reaction mixture containing 10 mM Hepes (pH 7.5), 10 mM KCl, 10 mM MgCl2, 1 mM DTT, 1 mM EDTA, 10% glycerol, 40 ng of biotin-labeled, double-stranded oligonucleotide, 1.5 μg of poly(dI-dC) (Amersham), and 60 μg of TISC nuclear lysate prepared using the NE-PER nuclear protein extraction reagent (Thermo Fisher) according to the manufacturer's guidelines. Reaction mixtures were incubated for 10 min at room temperature and subjected to electrophoresis on pre-run 5% native PAGE gels in 0.5× Tris/Borate/EDTA (TBE) buffer. Gels were transferred to nitrocellulose membranes and incubated with streptavidin-HRP (Cell Signaling Technology) for 3 h followed by extensive washing in Tris-buffered saline containing 0.1% Tween-20 and detection with the Supersignal West Pico Chemiluminescence kit (Pierce Biotechnologies).

Chromatin Immunoprecipitation Assays. $CD133^+$ liver TISCs grown in 10-cm cell culture dishes were fixed for 10 min at room temperature by addition of 1% paraformaldehyde to the growth medium. Cells were washed twice in cold PBS supplemented with complete protease inhibitor mixture and gently scraped from the plate. Cell lysis and chromatin immunoprecipitation (ChIP) were performed using the ChIP Assay Kit (Millipore). For chromatin fragmentation, cells were sonicated using a Branson Sonifier 450 on power setting 4 in 30-s bursts with 1 min cooling on ice for a total sonication time of 4 min. For immunoprecipitations, 8 μg of each antibody was used.

Tumor Implants. $Lepr^{db/db}$, *ob/ob*, and NOG mice were purchased from Jackson Laboratories and housed under pathogen-free conditions in accordance with approved Institutional Animal Care and Use Committee protocols. Liver TISCs (4×10^4) were resuspended in 100 μL of 50% Matrigel (BD Biosciences) with PBS and injected s.c. into the dorsal hind flanks of anesthetized mice. Tumors were measured with calipers and the volume calculated according to the formula $V = a \times b^2/2$, where a represents the largest and b the smallest superficial diameters (5).

Statistical Analysis. Statistical significance was estimated by unpaired, two-tailed Student's t test. P values are indicated in the figures. Bars represent the mean and error bars the SD. For all figures, statistical significance is represented by asterisks above each column: $^*P < 0.05$ and $^{**}P < 0.01$.

For gene expression meta-analysis, microarray expression data corresponding to patient samples from pancreatic cancer (GSE1542), breast cancer (GSE5764), and multiple myeloma (GSE5900) as well as CaCo-2 human epithelial colorectal adenocarcinoma cells (GSE24747) were obtained from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo). The correlation in gene expression between OB-R and POU5F1(OCT4) was determined with Pearson's correlation coefficient using the R/Bioconductor software suite.

1. Takahashi K, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.
2. Catena R, et al. (2004) Conserved POU binding DNA sites in the Sox2 upstream enhancer regulate gene expression in embryonic and neural stem cells. *J Biol Chem* 279:41846–41857.
3. Zappone MV, et al. (2000) Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. *Development* 127: 2367–2382.
4. Machida K, et al. (2009) Toll-like receptor 4 mediates synergism between alcohol and HCV in hepatic oncogenesis involving stem cell marker Nanog. *Proc Natl Acad Sci USA* 106:1548–1553.
5. Carlsson G, Gullberg B, Hafström L (1983) Estimation of liver tumor volume using different formulas: An experimental study in rats. *J Cancer Res Clin Oncol* 105:20–23.

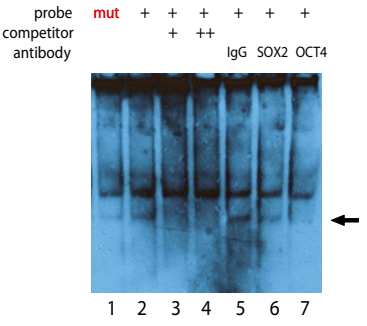

Fig. S1. Electrophoretic mobility shift assay. A 5′ biotinylated, double-stranded oligonucleotide probe corresponding to the candidate OCT4/SOX2 recognition site in *Lepr* (lane 2) or a mutated site harboring both sets of *Mut1* and *Mut2* mutations (lane 1) was incubated with $CD133^+$ TISC nuclear extracts. A 10- or 50-fold excess of unlabeled competitor probe was added in lanes 3 and 4, respectively. Control IgG (lane 5) or antisera specific for SOX2 (lane 6) or OCT4 (lane 7) were added to incubations before resolving by native gel electrophoresis.

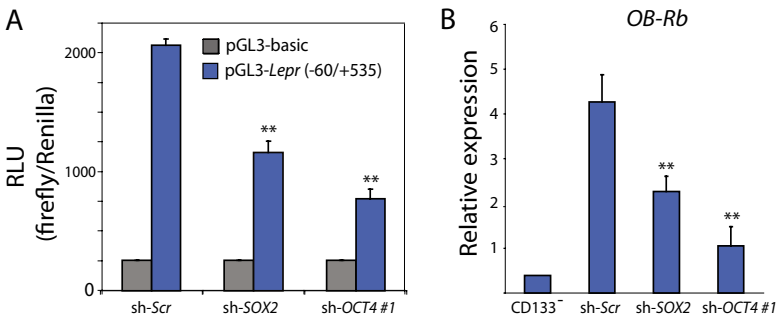

Fig. S2. Depletion of OCT4 or SOX2 reduces activity of the *Lepr* promoter. (*A*) Depletion of SOX2 or OCT4 attenuates activity of the *Lepr* luciferase reporter. TISCs stably transduced with the indicated lentiviral shRNA vectors were transfected with either the empty reporter vector (pGL3-Basic) or with the *Lepr* (−60/+535) luciferase reporter. Forty-eight hours after transfection, the normalized luciferase activity was determined. Asterisks (**) indicate a significant ($P < 0.01$) decrease relative to sh-*Scr* control. (*B*) Quantitative RT-PCR analysis of endogenous mRNA transcript levels for the leptin receptor (*OB-R*) in $CD133^-$ controls and in TISCs stably expressing a nontargeting, scrambled shRNA (sh-*Scr*) or in shRNAs targeting *SOX2* or *OCT4*. $^{**}P < 0.01$ relative to *sh-Scr* control.

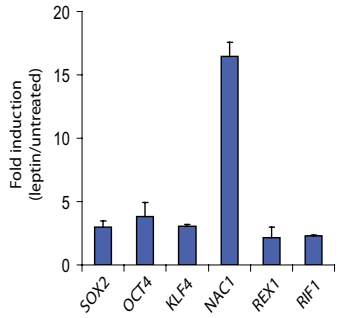

Fig. S3. Leptin induces multiple components of the pluripotency-associated TF network in TISCs. TISCs were untreated or exposed to leptin (150 ng/mL) for 48 h, harvested, and analyzed by qRT-PCR to evaluate the expression of the indicated genes. Error bars represent SD from three independent biological replicates.

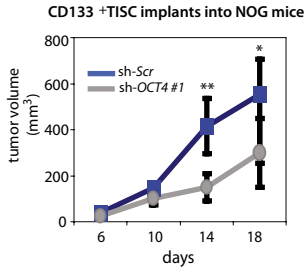

Fig. S4. Knockdown of OCT4 attenuates the growth of $CD133^+$ TISC tumors in NOG mice. TISCs stably transduced with nontargeting control shRNA (sh-*Scr*) or shRNA targeting *OCT4* (sh-*OCT4 #1*) were implanted into the dorsal hind flanks of NOG mice at 4×10^4 cells per implant ($n = 8$/group). Tumor growth was measured by using calipers on the indicated days. Data are representative of two independent experiments. $^*P < 0.05$; $^{**}P < 0.01$.