Supporting Information

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SI Experimental Procedures

Cell Culture and Cloning. Clonal epithelial and mesenchymal cell lines were newly established from oviducts of $p53^{-/-}$ mice on embryonic day 18 (E18; a presumptive oviduct in a Müllerian duct) and postnatal day 3 (P3). Dissected tissues were minced with razorblades and plated on 100-mm culture dishes containing 10% FCS. Pieces of minced oviducts attached to culture dishes after incubation for 24–36 h. After 5 days of culture, the attached organoids spread and formed monolayers. Cloning was started with the same methods as described previously (1). Two epithelial cell lines (E1 and B1) were established from an oviduct on E18, and two mesenchymal cell lines (S1 and S10B) were from an oviduct on P3. They were maintained in 10% FCS. Three clonal cell lines established from adult oviducts of $p53^{-/-}$ mice (1) were used in this study. AB and 6B were epithelial cell lines, and S3B was a mesenchymal cell line.

Immunohistochemistry. The cryosections on slides were rinsed with PBS and permeabilized in 0.2% Triton X-100/PBS for 5 min. The sections were incubated for 1 h at room temperature with primary antibodies. The primary antibodies were anti-Ovgp1 polyclonal antibody (oviductin, 1/200; Santa Cruz Biotechnology), anti-β-tubulin IV monoclonal antibody (tubb4, 1/100; Boeringer Mannheim Biochemica), anti-α-actin antibody (Progen Biotechnik), and rabbit antiserum raised against Fstl1. After washing three times with cold PBS, the slides were incubated with fluorescein-isothiocyanate–conjugated anti-mouse IgG serum (Biosource International) or Cy3-conjugated anti-rabbit IgG serum (Invitrogen) at room temperature for 2 h. On control slides, incubation with the primary antibodies was omitted.

Recombinant Tissue Preparation and Grafting Under the Kidney Capsule. The method was further modified for tissue cells recombinant in the present study. Oviducts were dissected from neonatal CD1 mice. They were cut into three tubes (infundibulum, ampulla, and isthmus), placed into 1% trypsin, and incubated at 4 °C for 90 min. Then epithelia were squeezed out from the tubes with fine surgical forceps. Vaginas were bisected into halves, and epithelia and mesenchyme were separated as described previously (2). To prepare recombinant tissue, a mesenchymal tissue was injected with a 0.1- to 10-μL pipette tip (Molecular Bio Products) into a 80-μL gel drop of Cellmatrix type I-A (gelatin from Nitta) on a siliconized dish. An epithelial tissue or cells of epithelial cell lines (5×10^8 cells/mL) in 0.1–0.2 μL were injected into an area adjacent to the mesenchymal tissue in matrix. The matrix drop containing epithelial cells and a mesencymal tissue was incubated for 5 min at 37 °C, placed on a cell culture insert, and cultured for 2 days in 10% FCS at 37 °C in a humidified atmosphere of 5% CO_2. After the incubation, recombinant tissues were grafted under the kidney capsule of adult female CD1. Four weeks after grafting, animals were killed and recombinant tissues were harvested. Recombinants of tissues derived from CD1 mice and cells derived from $p53^{-/-}$ mice might induce immunoreaction against allografts when grafted under the kidney capsule of CD1 mice. In the preliminary experiment, recombinants prepared with tissues of CD1 mice and cell lines established from $p53^{-/-}$ mice were grafted under the kidney capsule of CD1 and nude mice for 4 weeks. Although the success rate of oviduct reconstruction was 60% in nude mice, it was 40% in CD1 mice. No distinguishable differences in histoarchitecture were recognized among reconstructed oviducts in two groups of recipients. From a practical point of view, the experimental paradigm (CD1 mice as recipients, drafting site, and period) was reasonable. Therefore, recombinants were grafted in CD1 mice.

Ratio of Ciliated Cells and Secretory Cells. To determine the ratio of ciliated cells (β-tubulin IV-positive) and secretory cells (Ovgp1-positive), numbers of β-tubulin IV-positive epithelial cells, Ovgp1-positive epithelial cells, and double-negative epithelial cells were counted on the screen in three frames for each specimen (more than 400 total epithelial cells) with an AxioCAM MRm (Carl Zeiss) interfaced with an Axiovert 200M (Carl Zeiss). Results were based on analysis of 8–24 tissue recombinants per group. Data were analyzed by *t* test or ANOVA test. A statistically significant difference was defined as $P < 0.05$.

Coculture of Epithelial and Mesenchymal Cells. The medium was DMEM/F12 without phenol red (Sigma) containing heat-inactivated FCS at 10% (10% FCS; Sigma) supplemented with penicillin (31 μg/mL; Sigma), streptomycin (50 μg/mL, Sigma). Epithelial and mesenchymal cells were cocultured separately by a culture insert filter (Millicell-PCF filter six-well plate inserts, 0.4-μm pore; Millipore). Epithelial cells were seeded onto the inside of an insert above the membrane at a density of 2×10^5 cells/well, and mesenchymal cells were plated onto the bottom of a six-well plate at a density of 5×10^5 cells/well. Cells became confluent in 24 h in 10% FCS, and coculture was started by setting the insert on the six-well plate and terminated 24 h later.

After coculture with the neonate-derived mesenchymal cells, epithelial cells were passaged at a density of 1×10^6 cells onto the bottom of a 60-mm dish as sublines. d-E1 (determined E1) was a subline of E1 cells cocultured with S1 cells. Sublines became confluent in 24 h, and 1×10^6 cells from the sublines were passaged in a 60-mm dish; the remaining cells were used for RNA isolation (monoculture for 1 day). The passaged cells were cultured in 10% FCS for 4 days and used for RNA isolation (monoculture for 5 days).

Real-Time RT-PCR. Total cellular RNA from cell lines was extracted, isolated, and purified using the SV total RNA kit (Promega). The reverse-transcriptase reactions were performed using first-strand syntheses kit (Amersham). Real-time PCR was performed using the ABI Prism 7000 detection system (Applied Biosystems) with SYBR-Green as fluorescent dye enabling real-time detection of PCR products according to the manufacturer's protocol.The cDNA was submitted to real-time PCR using the primer pairs as described below. Cycling conditions were 95 °C for 15 min, followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s. For quantification, the target gene was normalized to the internal standard gene GAPDH using the following equation: $T_0/R_0 = K(1 + E)^{CT,R-CT,T}$. T_0 is the initial number of target gene mRNA copies, and R_0 is the initial number of standard gene mRNA copies, and E is the efficiency of amplification. CT,T is the threshold cycle of the target gene, CT,R is the threshold cycle of the standard gene, and K is the constant. The efficiencies were shown to be in the range $0.90 < E < 1.0$. The following primer sets were derived from the GenBank sequences: *Ovgp1* (accession no. NM_007696, forward: 5′-TCT GCA GGA TGA AAA CGT TCT-3′; reverse: 5′-CCT CCA ATG GAC AGC AGT GT-3′), *Foxj1* (accession no. NM_008240, forward: 5′-GGA CAA CTT CTG CTA CTT CCG-3′; reverse: 5′-TCA AGG ACA GGT TGT GGC G-3′), *Fstl1* (accession no. NM_008047, forward: 5′-AGT CTG CGA GTC CAT CTG CC-3′; reverse: 5′-TGG ATG AGG CGC CGT C-3′), and GAPDH

(accession no. M17701, forward: 5′-TCT ACC CAC GGC AAG TTC AAT-3′; reverse: 5′-ACC CCA TTT GAT GTT AGC GG-3′).

Construction of cDNA Library for Signal Sequence Trap, a Retrovirus-Mediated Expression Screening Method. Total RNA was isolated from confluent cells, and poly(A) + RNA was further purified using an mRNA purification kit. cDNA was synthesized from poly (A) + RNA with random hexamers using the SuperScript Choice System (Invitrogen), separated through a SizeSep 400 Spun Column, and inserted into BstxI sites of the pMX-SST vector with the aid of BstxI adapters. The ligand DNA was electroporated into DH10B cells using Gene Pulser. The plasmid library was amplified in DH10B cells, and DNA was then purified using a plasmid isolation kit. High-titer retroviruses representing the signal sequence trap, a retrovirus-mediated expression screening (SST–REX) library, were produced using a packaging cell line Plat-E and were infected to Ba/F3 cells. After a 1-day infection period, the selection of factor-independent Ba/f3 cells was started in the absence of IL-3 using 96-well plates. Genomic DNA was extracted from Ba/F3 clones and subjected to PCR to recover integrated cDNA using a set of primers with vector sequences. The resulting PCR fragments were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit and were analyzed with an automatic sequencer. The resulting sequences were subjected to a database search against the GenBank DNA database using the Blast server.

Overexpression of Mesenchymal Factors in NIH/3T3 Cells. NIH/3T3 cells were stably transfected with each mesenchymal factor identified by SST screening. The cDNAs of mesenchymal factors were inserted into the mammalian expression vector pcDNA3.1/Hygro (Life Technologies).

Three microliters of Fugene6 (Roche) was added to 100 μl of Optimem I medium (Invitrogen) and mixed by vortexing. The mixture was then incubated for 5 min at room temperature. One microgram of the expression vectors was added to the tube and incubated for 15 min at room temperature. The transfection complex was added dropwise into medium. Cells were then incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. Control lines were generated by stably transfecting NIH/3T3 cells with pcDNA3.1/Hyg vector. Transfected clones were selected with hygromysin (500 μg/mL). S10B cells were likewise stably transfected with vector and with the mesenchymal factors. For each transfection (with empty vector, mesenchymal factor), 10–20 lines were isolated from single colonies. Cell lines were assayed for ectopic gene expression by real-time RT–PCR.

Immunoblotting and Immunoprecipitation. Solubilized samples (20 μg protein/lane) were electrophoresed in 7.5% SDS polyacrylamide–slab gel and electrophoretically transferred onto polyvinylidine difluoride membrane (Millipore). Membranes were incubated with anti-Fstl1 serum.

Immunoprecipitation assays were performed with protein G Sepharose (GE Healthcare) and subsequently analyzed by Western blot.

Anti-Fstl1 Antibody Production. To select the best peptides for antibody production, protein sequences of Fstl1 were analyzed by the set of algorithms. While noting the phosphorylated site and hydrophilic region, 15 amino acids of the N-terminal region (CGTAEKTKKVNTKEI) were selected for the antigen. New Zealand White rabbits were injected with the synthetic peptides. Primary immunization was performed with 0.50 mg MAP or 0.25 mg keyhole limpet hemocyanin (KLH) emulsified with Freund's complete adjuvant, and boosters were with 0.50 mg MAP or 0.10 mg KLH emulsified with Freund's incomplete adjuvant. After the injection of the fourth booster, large-volume production bleeds (~50 mL/rabbit) were obtained through a serum collection, and then titer and specificity of the antiserum were tested by ELISA.

In Situ Hybridization. Oviducts of adult and perinatal mice were embedded in Tissue-Tek OCT compound (Sakura Finetechnical) and frozen. Cryosections (8 μm) were made with a Jung Frigocut 2800E (Leica) and then mounted on SILANE-PREP slides (Sigma). Digoxgenin-labeled probes for specific transcripts were produced with the digoxigenin RNA labeling kit (Roche) and prepared by PCR with primers designed using published sequences. A 350-bp probe against *Fstl1* was generated from mouse oviduct cDNA using primers based on the *Fstl1* sequence (GenBank accession no. NM_008047, forward: 5′-GAG AGC TCA CAG CAG CAA TG-3′; reverse: 5′-GAG AGC TTG CCG TCT TGT TC-3′). Resultant bands were subcloned into pGEM T-easy vector (Promega) and sequenced to confirm sequence identity.

Hybridization was carried out at 55 °C for 16 h with hybridization buffer containing 50% formamide, 0.01 M Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% SDS, 0.6 M NaCl, 1× Denhardt's solution, 10% dextran sulfate, and 400 μg/mL yeast tRNA. The final wash was in 0.1× SSC at 55 °C for 1 h. Samples were incubated for 2 h with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (Roche) at 1:2000, washed, and processed for colorimetric detection using nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolylphosphatase p-toluidine salt.

1. Umezu T, Hanazono M, Aizawa S, Tomooka Y (2003) Characterization of newly established clonal oviductal cell lines and differential hormonal regulation of gene expression. *In Vitro Cell Dev Biol Anim* 39:146–156.

2. Bigsby RM, Cooke PS, Cunha GR (1986) A simple efficient method for separating murine uterine epithelial and mesenchymal cells. *Am J Physiol* 251:E630–E636.

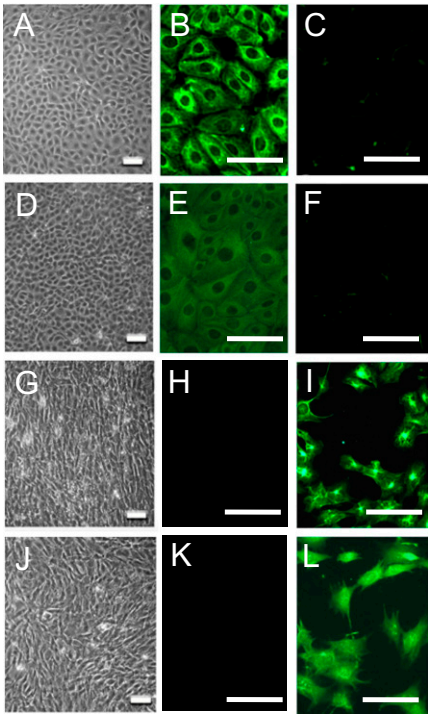

Fig. S1. Characterization of clonal cell lines established from perinatal oviducts. (*A*) E1 cells established from E18 oviducts were polygonal and had a smooth and round edge. They were positive for an epithelial marker CK 18 (cytokeratin 18) (*B*) and negative for a mesenchymal marker VT (vimentin) (*C*). (*D*) B1 cells established from E18 oviducts had a round and obscure edge. They were positive for CK 18 (*E*) and negative for VT (*F*). S1 cells established from P3 oviducts became overlapped as cell density increased (*G*). They were negative for CK 18 (*H*) and positive for VT (*I*). (*J*) S10B cells established from P3 oviducts were flat and did not overlap. They were negative for CK 18 (*K*) and positive for VT (*L*).

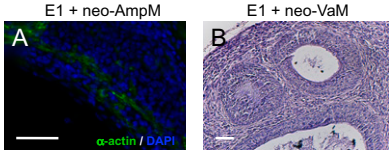

Fig. S2. Architecture of oviduct and vagina developed from recombinants. (*A*) Immunostaining for α-actin of recombinants of E1 cells with neo-AmpM. Muscle layers surround mesenchyme. (*B*) E1 cells recombined with mesenchymal tissues of vagina at P3 (neo-VaM) developed stratified epithelia.

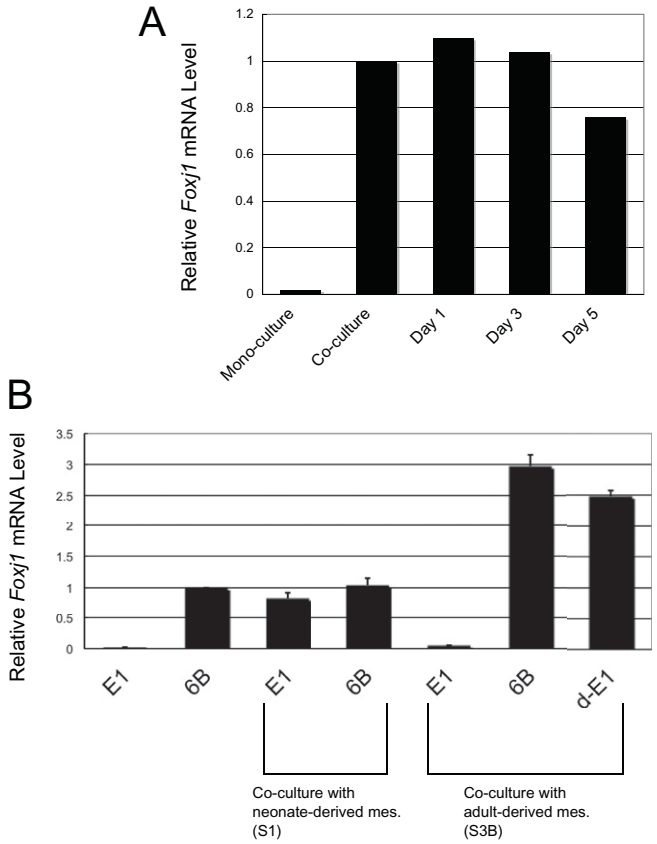

Fig. S3. *Foxj1* expressions of E1 cells cocultured with mesenchymal cell lines. (*A*) E1 cells cocultured with S1 cells were passaged and monocultured as a subline of E1 (d-E1) and subjected to quantitative PCR (q-PCR) analysis of *Foxj1* expression at the indicated days. (*B*) Epithelial cells (E1, d-E1, 6B: cilial type) and mesenchymal cells were cocultured and subjected to q-PCR analysis of *Foxj1* expression. Adult-derived mesenchymal cells (S3B) were capable of enhancing *Foxj1* expression on adult-derived cilial epithelial cells (6B) (1) . *Foxj1* expression of d-E1 cells was significantly increased as observed in 6B cells cocultured with S3B cells. Error bars indicate standard deviation of triplicate reactions. Error bars show the standard deviation (n = 3).

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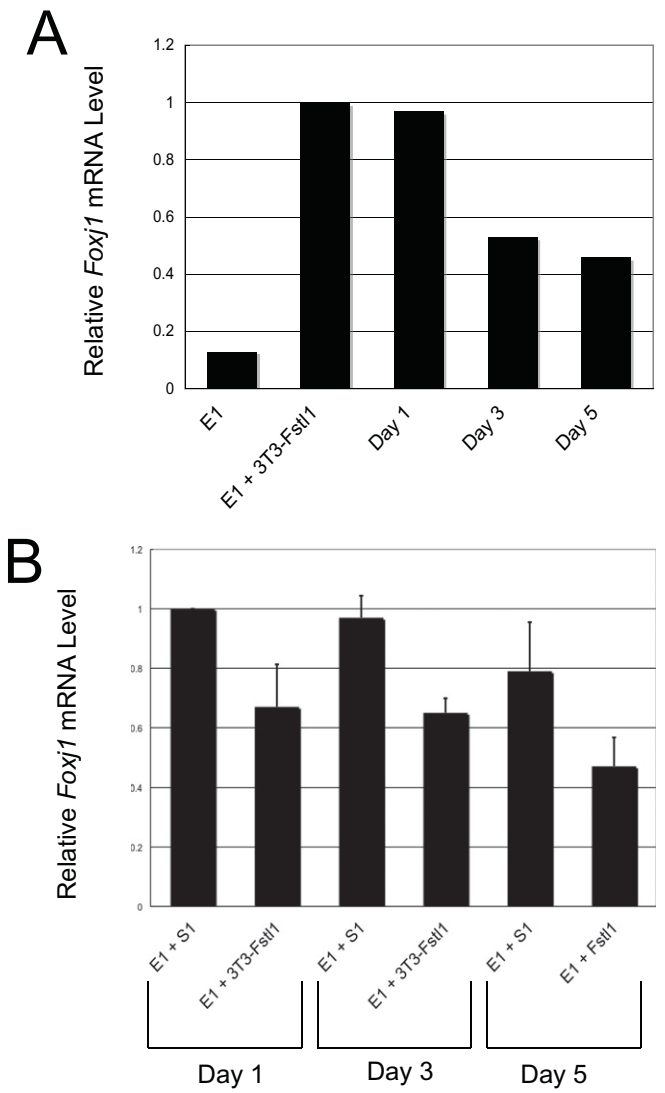

Fig. S4. Long-lasting effect of Fstl1 in coculture. (*A*) After cocultured with Fstl1-overexpressing cells for 24 h, E1 cells were monocultured for 5 days and subjected to real-time RT–PCR analysis of *Foxj1* expression. E1 cells without Fstl1-overexpressing cells continuously expressed *Foxj1*. (*B*) E1 cells were cocultured with Fstl1-overexpressing mesenchymal cells for 1, 3, or 5 days and subjected to real-time RT–PCR analysis of *Foxj1* expression. Error bars indicate standard deviation of triplicate reactions. Error bars show the standard deviation (n = 3).

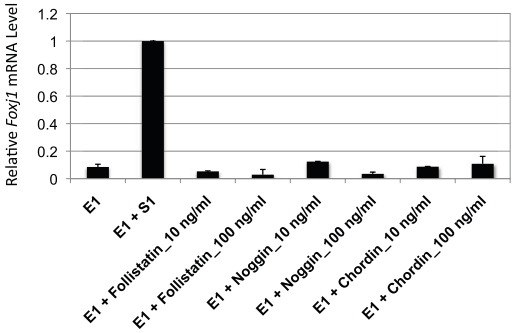

Fig. S5. *Foxj1* expressions of E1 cells monocultured with bone morphogenic protein (BMP) antagonists. E1 cells were monocultured for 24 h in 10% FCS + Follistatin (BioVision; 10 ng/mL, 100 ng/mL), or + Chordin (R&D Systems; 10 ng/mL, 100 ng/mL), or + Noggin (BioVision; 10 ng/mL, 100 ng/mL). RNAs were isolated from E1 cells and subjected to real-time RT–PCR analysis of *Foxj1* expression. *Foxj1* expression was induced in E1 cells when cocultured with S1 cells. *Foxj1* expression was not detected in E1 cells when monocultured in medium supplemented with BMP antagonists. Error bars indicate standard deviation of triplicate reactions. Error bars show the standard deviation (n = 3).

Table S1. Candidates of mesenchymal diffusible factors identified by SST method

Gene symbol	Gene full name
Grn	Granulin
Ifnar2	IFN (α and β) receptor 2
Mfge8	Milk fat globule-EGF factor 8 protein
Pla2g7	Phospholipase A2
Pdia3	Protein disulfide isomerase associated 3
Fstl1	Follistatin-like-1
Serpinh1	Serine (or cysteine) peptidase inhibitor, clade H

Seven clones were selected as candidates of mesenchymal diffusible factors determining epithelial cell fate.