Primary culture and immortalization of human fallopian tube secretory epithelial cells

Alison M Karst[1] & Ronny Drapkin[1,2]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA. [2]Department of Pathology, Division of Women's and Perinatal Pathology, Brigham and Women's Hospital, Boston, Massachusetts, USA. Correspondence should be addressed to R.D. (ronny_drapkin@dfci.harvard.edu).

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Primary human fallopian tube secretory epithelial cell (FTSEC) cultures are useful for studying normal fallopian tube epithelial biology, as well as for developing models of fallopian tube disease, such as cancer. Because of the limited ability of primary human FTSECs to proliferate *in vitro*, it is necessary to immortalize them in order to establish a cell line that is suitable for long-term culture and large-scale *in vitro* experimentation. This protocol describes the isolation of FTSECs from human fallopian tube tissue, conditions for primary FTSEC culture and techniques for establishing immortal FTSEC lines. The entire process, from primary cell isolation to establishment of an immortal cell line, may take up to 2 months. Once established, immortal FTSECs can typically be maintained for at least 30 passages.

INTRODUCTION

Immortal cell lines derived from healthy human tissue are fundamental tools for experimental cancer research. They are frequently used to characterize the phenotypic and genotypic differences between normal and malignant cells *in vitro* and are an excellent tool for cellular transformation studies. Our laboratory recently published a report describing the immortalization and transformation of primary human FTSECs[1], a cell type that previously received little attention in the cancer research arena. An urgent need for research into fallopian tube–derived cancer has recently been realized with the discovery that many pelvic tumors thought to be ovarian in origin may have actually arisen from the fallopian tube. The most lethal ovarian cancer among American women is high-grade serous ovarian carcinoma (HGSOC). This tumor type is conventionally thought to arise from the ovarian surface epithelium, because the bulk of the tumor typically involves the ovary. However, new data suggest that HGSOC may actually begin in the distal fallopian tube (located immediately next to the ovarian surface) and subsequently spread to the ovary[2–5]. The presumptive cell of origin for fallopian tube–derived serous tumors is the FTSEC. To study the transformation of this cell type and examine how it may contribute to HGSOC development, it is critical that experimental models of the fallopian tube be developed. Whether all HGSOCs arise from the fallopian tube is not yet clear. Therefore, it is important not to discount the possibility that both the fallopian tube and ovary may serve as sites of serous tumorigenesis. In this context, fallopian tube–based experimental models are an essential complement to preexisting ovarian-based models for the comprehensive study of pelvic serous cancer development.

The hypothesis that most high-grade pelvic serous tumors begin in the fallopian tube is creating a paradigm shift in our understanding of HGSOC pathogenesis, and it has substantial clinical implications for screening and prevention[4,6–12]. First, the methods currently used for early detection of this cancer, which focus primarily on the ovary, are largely ineffective. This has resulted in late-stage diagnoses and strongly contributes to the low 5-year survival rate of only 30% (SEER Cancer Statistics Review, 1975–2004, National Cancer Institute; http://seer.cancer.gov/csr/1975_2004/). It is quickly becoming apparent that screening efforts must be shifted to the fallopian tube in order to reduce HGSOC mortality. Second, women with germline *BRCA1* mutations, who are genetically predisposed to breast and ovarian malignancies, frequently elect to undergo prophylactic salpingo-oophorectomy (ovary and fallopian tube removal) to reduce their risk of developing cancer. If fallopian tubes are indeed the primary source of HGSOC, it may be possible to reduce risk by removing the fallopian tubes alone, thus preserving fertility and avoiding the morbidity associated with premature menopause[11].

These recent advances in our understanding of HGSOC pathogenesis and their potential clinical impact have sparked an unprecedented interest in fallopian tube epithelial biology and a high demand for experimental tools with which to study fallopian tube transformation. To address this need, our laboratory recently developed two alternate methods for culturing primary human fallopian tube cells: an *ex vivo* model of the fallopian tube epithelium[13,14] and an *in vitro* model of FTSECs that is based on primary cell immortalization[1,15] (**Fig. 1**). The two models differ in that the *ex vivo* model intends to recapitulate a 3D polarized epithelium consisting of multiple epithelial cell types, whereas the FTSEC model aims to isolate a pure population of secretory epithelial cells in order to generate immortal FTSEC lines suitable for long-term *in vitro* culture. Our protocol for the *ex vivo* culture of primary fallopian tube epithelial cells has recently been published elsewhere[14]. Here we present our protocol for establishing primary immortal FSTEC lines. As both of our models require freshly collected primary human fallopian tube epithelial cells, the initial steps of tissue processing (Steps 1–7 of the present protocol) are very similar in both protocols. However, the two protocols diverge markedly after the epithelial dissociation step. Immortal cell lines established by the following procedure may be used to represent 'normal' FTSECs in a myriad of *in vitro* experiments. They may also be used for transformation assays in which immortal FTSECs are transduced with different genetic alterations in order to systematically query the functional consequences of specific genetic changes and assess their respective contributions to cellular transformation[1]. This is especially useful now that large-scale genomic analyses of HGSOC have been published by The Cancer Genome Atlas and other groups[16–22] and tools for the experimental validation of these data are critically needed. Developing immortal cell lines enables us to study the effects of HGSOC genomic

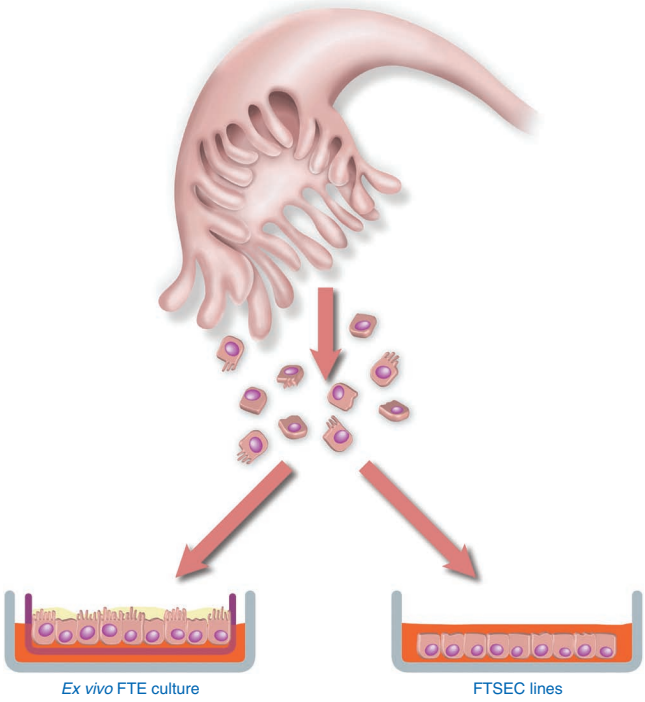

Figure 1 | Methods for culturing primary human fallopian tube epithelial cells. Epithelial cells isolated from primary human fallopian tube tissue may be cultured using one of two methods. Shown on the left, *ex vivo* cultures are used to model the fallopian tube epithelium (FTE). Here, epithelial cells are dissociated from fallopian tube tissue and seeded onto a porous membrane, where both the ciliated and secretory cell types assemble in a polarized manner, thus recreating an intact FTE. This is a small-scale model best suited for short-term experiments examining epithelial biology. Shown on the right are FTSEC lines that are used to study the secretory cell type, which represents the presumptive cell of origin of high-grade pelvic serous carcinomas. For this model, FTSECs are immortalized *in vitro* to establish cell lines that can be expanded and used for large-scale experiments, such as transformation and tumor xenograft studies.

aberrations on nontransformed FTSECs and to construct genetically defined human xenograft models of fallopian-derived cancer[1]. Such models are invaluable for the study of high-grade serous tumor biology, as well as for testing novel therapeutic agents.

Experimental design

Tissue acquisition. This protocol requires collection of a fresh normal human fallopian tube. Access to fresh tissue should be coordinated through a hospital pathology department or tissue bank service with approval from the Institutional Review Board. It is important to be aware that the approval process may take anywhere from several weeks to months.

Retroviral transduction with defined genetic alterations

Primary human FTSECs grown on plasticware will typically senesce within 2–3 passages and therefore must be immortalized to facilitate expansion and long-term culture. FTSEC immortalization can be readily achieved by expressing human telomerase reverse transcriptase (hTERT) and perturbing the p53 and pRb tumor suppressor pathways. In this protocol, we use shRNA to silence p53 expression and mutant CDK4^{R24C} to inhibit pRb activity. However, a variety of equivalent genetic elements may be used to obtain similar results. For example, SV40T antigens will perturb both the p53 and pRb pathways[1,23] or a dominant-negative mutant form of p53 may be used instead of p53 shRNA. This protocol uses retroviruses to stably express hTERT, p53 shRNA and CDK4^{R24C} in primary FTSECs. Retroviral expression vectors available from Addgene can be used to generate retroviruses by standard techniques (see REAGENTS and REAGENT SETUP).

It is important that primary FTSECs be replicating during retroviral infection in order to achieve high-efficiency gene transfer[24]. Primary FTSECs proliferate quickly when first plated, but they do not always grow well after they are split. We therefore recommend transducing FTSECs as soon as they reach ~50% confluency, before they are passaged. However, primary FTSEC growth dynamics are highly sample dependent. In some cases, the cells continue to grow well after the first passage, and thus the cell population may be expanded before transduction. If a sample shows rapid growth and/or becomes 100% confluent before there is an opportunity to transduce the cells, it is reasonable to split the cells at a ratio of 1:2 before performing the transduction, as described in the PROCEDURE.

Maintenance of immortal FTSEC lines

We recommend supplementing FTSEC medium (FTM) with Ultroser G (USG) serum substitute throughout this protocol, because we find that naive and newly immortalized FTSECs grow better in the absence of serum. However, once an immortal FTSEC line has been established and low passages have been safely preserved, USG may be replaced with 10% (vol/vol) fetal bovine serum (FBS), which is more economical. In most cases, immortal FTSEC lines adapt well when switched to serum-containing medium. However, it is best to test this on a small plate of cells before making any changes to the culture conditions.

MATERIALS

REAGENTS

- Human fallopian tube tissue **! CAUTION** Human tissue collection requires patient consent and must be approved by the local Institutional Review Board. **! CAUTION** Human tissue and blood are biohazards. Handling and disposal of these materials must be carried out in accordance with institutional biohazard safety regulations.
- PBS, sterile (Mediatech, cat. no. 21-040)
- Minimum essential medium (MEM) with Earle's salts and L-glutamine (Mediatech, cat. no. 10-090)
- Pronase from *Streptomyces griseus* (Roche, cat. no. 10165921001)
- Deoxyribonuclease I (DNase) from bovine pancreas (Sigma-Aldrich, cat. no. DN25)
- Human placental collagen, Bornstein and Traub Type IV (Sigma-Aldrich, cat. no. C7521)
- Glacial acetic acid (Fisher Scientific, cat. no. BP2401500) **! CAUTION** Glacial acetic acid is corrosive. Wear eye and skin protection when handling it.
- Ultroser G serum substitute (USG; Pall, cat. no. 15950-017), distributed in the USA by Crescent Chemical Company (cat. no. 67042) **▲ CRITICAL** Pall is the sole manufacturer of USG. Although other serum substitute formulations are commercially available, none has been tested by our lab for use in this protocol.
- Water, sterile cell culture grade (Mediatech, cat. no. 25-055)
- Water, double distilled (ddH_2O)

- Dulbecco's modification of Eagle's medium/Ham's F-12 50/50 mix (DMEM-Ham's F12) without L-glutamine (Mediatech, cat. no. 15-090)
- Penicillin-streptomycin (Gibco, cat. no. 15140)
- Replication-defective vesicular stomatitis virus G glycoprotein (VSVG)-pseudotyped retroviruses encoding hTERT, p53 shRNA and CDK4^{R24C} under CMV promoter control. Retroviral expression vectors are available from Addgene (pBabe-neo-hTERT, cat. no. 1774; pMKO.1-puro-p53 shRNA, cat. no. 10671; and pBabe-hygro-CDK4^{R24C}, cat. no. 11254). Standard protocols for retrovirus production are widely available and accessible through the Addgene website
- Polybrene (hexadimethrine bromide; Sigma-Aldrich, cat. no. H9268)
- Sodium chloride (Sigma-Aldrich, cat. no. S5886)
- Trypsin-EDTA, 0.25% (wt/vol) (Gibco, cat. no. 25200)
- Trypsin neutralizing solution (TNS; Lonza, cat. no. CC-5002)
- G418 (optional; InvivoGen, cat. no. ant-gn-1)
- Puromycin (InvivoGen, cat. no. ant-pr-1)
- Hygromycin B (optional; InvivoGen, cat. no. ant-hm-1)
- DMSO (Sigma-Aldrich, cat. no. D2650)
- p53 antibody (Santa Cruz Biotechnology, cat. no. sc-81168)
- CDK4 antibody (Epitomics, cat. no. 2341-1)
- PAX8 antibody (ProteinTech Group, cat. no. 10336-1-AP)
- CK7 antibody (Epitomics, cat. no. 2303-1)
- GAPDH antibody (Cell Signaling Technology, cat. no. 2118)
- RB antibody (Cell Signaling Technology, cat. no. 9309)
- Phospho-Rb (Ser780) antibody (Cell Signaling Technology, cat. no. 8180)
- FOXJ1 antibody (Abcam, cat. no. ab40869)
- Liquid nitrogen

EQUIPMENT

- Stainless steel forceps (Roboz, cat. no. RS-5040)
- Glass beaker, 200-ml capacity
- Petri dish, 100-mm, sterile (BD Falcon, cat. no. 351029)
- Disposable scalpel (BD Bard-Parker, cat. no. 372615)
- Centrifuge tubes, sterile, 50 ml (Corning, cat. no. 430829)
- Centrifuge tubes, sterile, 15 ml (Corning, cat. no. 430829)
- Laboratory rocker
- Temperature-controlled centrifuge (Eppendorf, model 5180R or equivalent) with a swing bucket rotor suitable for 15-ml tubes, 50-ml tubes and tissue culture plates
- Disposable serological pipettes, 1 ml (Corning, cat. no. 4485)
- Disposable serological pipettes, 2 ml (Corning, cat. no. 4486)
- Disposable serological pipettes, 5 ml (Corning, cat. no. 4487)
- Disposable serological pipettes, 10 ml (Corning, cat. no. 4488)
- Bottle-top vacuum filter system (0.22 µm), 250-ml capacity (Corning, cat. no. 431096)
- Bottle-top vacuum filter system (0.22 µm), 500-ml capacity (Corning, cat. no. 431097)
- Combination hot plate/magnetic stirrer
- Magnetic stir bar
- Hemocytometer
- Light microscope
- Cell culture CO_2 incubator
- Cell culture plates, 24 well (Corning, cat. no. 3516)
- Cell culture plates, 12 well (Corning, cat. no. 3513)
- Cell culture plates, six well (Corning, cat. no. 3526)
- Cell culture dish, 100 mm (Corning, cat. no. 430167)
- Cryogenic vials, 1.8-ml capacity (Thermo Scientific, cat. no. 375418)

REAGENT SETUP

Fallopian tube sample collection Collect fallopian tubes from patients undergoing surgery for benign gynecological indications such as fibroids, ovarian cysts, hysterectomy or alternate conditions not affecting the fallopian tubes. The fallopian tube specimen must be healthy and not associated with gynecological malignancy. Upon surgical excision, the fallopian tube should be immersed in sterile PBS and kept on ice. The most plentiful source of epithelial cells is the distal (fimbrial) region of the fallopian tube. Therefore, it is important that the integrity of the fimbrial region be preserved during specimen collection. The tissue should be picked up and transported to the laboratory as soon as possible, ideally within a few hours.

Dissociation medium Dissolve 350 mg of Pronase and 25 mg of DNase in 250 ml of MEM. Mix until all solid material is dissolved. Filter-sterilize the solution using a 250-ml capacity 0.22-µm bottle-top vacuum filter. Store at 2–8 °C for up to 4 weeks.

Ultroser G Ultroser G is supplied as a lyophilized powder and must be reconstituted before use. Add 20 ml of sterile water to one 20-ml bottle of USG and wait until the material is fully dissolved. This may take up to 20 min. Store unused reconstituted USG at −20 °C for up to 6 weeks. Do not freeze and thaw more than once. USG may be filtered (0.22 µm) if desired without loss of activity, although it is not necessary if it is prepared using aseptic techniques.

FTM Under sterile conditions, combine the following reagents: 485 ml of DMEM-Ham's F12, 10 ml of reconstituted USG and 5 ml of penicillin-streptomycin. Store at 2–8 °C for up to 4 weeks. Warm to 37 °C before use.

Human placental collagen solution Add 100 ml of ddH_2O to a small beaker. By using forceps, weigh out 30 mg of human placental collagen fibers and place them on top of the water. Pipette 100 µl of glacial acetic acid directly onto the collagen to help it dissolve. Add a small magnetic stir bar to the beaker. By using a combination hot plate/magnetic stirrer, warm the solution to 37 °C and stir it for 30 min or until the collagen is completely dissolved. Remove the solution from heat and dilute it to 500 ml with ddH_2O. Filter-sterilize the solution using a 500-ml capacity 0.22-µm bottle-top vacuum filter. Store at 2–8 °C for up to 6 months.

Collagen-coated tissue culture plates Under sterile conditions, pipette a sufficient volume of human placental collagen solution into the wells of tissue culture plates to cover the bottom of the wells. For a 24-well plate, add 250 µl per well; for a 12-well plate, add 500 µl per well; and for a 6-well plate, add 1 ml per well. Ensure that the entire surface of each well is covered. Stack plates in a sealed container to keep them sterile and store them at room temperature (20–25 °C) for up to 6 months. Immediately before use, rinse the wells with sterile PBS to remove any excess collagen solution.

Polybrene Prepare a solution of 1 µg ml^{-1} Polybrene in 0.9% (wt/vol) NaCl. Filter-sterilize or autoclave the solution before use. Store the solution at 2–8 °C for up to 1 year.

Retroviruses Retroviral supernatants produced using standard techniques (by transfection of 293T cells) typically have titers of 10^5–10^6 infectious units per ml. Although unconcentrated viral supernatants may be used to infect primary FTSECs, it is best to concentrate viruses as much as possible in order to achieve higher transduction efficiencies. There are several options including ultracentrifugation, use of a virus concentration kit or having the virus prepared by a viral vector core. To concentrate by ultracentrifugation, spin the viral supernatant at 20,000*g* for 1 h at 4 °C[25]. Discard the supernatant and resuspend the virus in a small amount of MEM overnight at 2–8 °C. To achieve a 500× increase in viral titer, for example, spin down 40 ml of viral supernatant and resuspend the virus in 80 µl of MEM. Alternatively, there are commercial virus concentration kits that are able to concentrate retroviral supernatants 100–500×, or many institutions have viral vector core facilities offering retrovirus production services. Such viral vector cores typically provide purified virus with titers ≥10^8 infectious units per ml, as quantified by Southern blot analysis.

PROCEDURE

Isolation of fallopian tube secretory epithelial cells

1| Rinse the fallopian tube with PBS to wash away excess blood and then transfer it to a 100-mm Petri dish using forceps (**Fig. 2a**,**b**).

! CAUTION This and all subsequent steps should be carried out in a Biosafety Level 2 cabinet under sterile conditions.

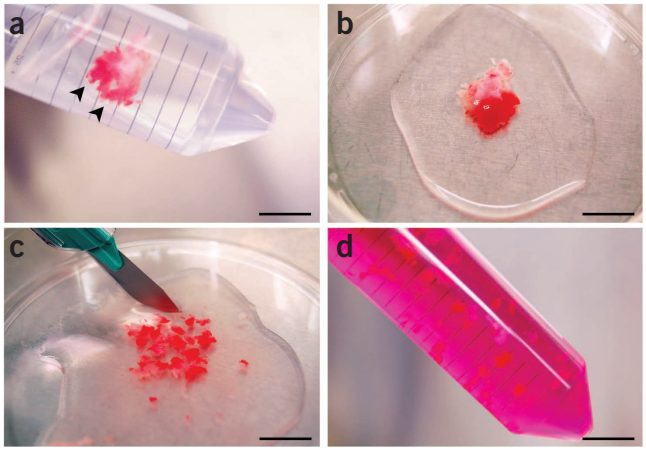

Figure 2 | Fallopian tube tissue processing. (**a**) Intact fallopian tube with prominent fimbria (arrowheads), immersed in PBS. (**b**) Appearance of fallopian tube after transfer to a Petri dish. (**c**) Mincing of fallopian tube tissue. (**d**) Minced tissue suspended in dissociation medium. Scale bars, 10 mm.

2| Cut off and discard any connective tissue using a scalpel. Next, mince the fallopian tube and transfer the minced tissue to a 50-ml centrifuge tube containing 45 ml of cold dissociation medium (**Fig. 2c**,**d**). Cap the tube tightly and place it on a laboratory rocker at 2–8 °C. Rock gently for 36–48 h. During this step, epithelial cells are enzymatically dissociated from the bulk tissue.

3| Invert the tube once or twice to resuspend the minced tissue and then hold the tube upright, allowing bulky pieces to settle to the bottom. Immediately decant the supernatant (containing dissociated epithelial cells) into a second 50-ml centrifuge tube. Pellet the decanted epithelial cells by centrifugation at 200*g* for 5 min at 20–25 °C. Discard the supernatant.

4| To increase the yield of dissociated cells, add 45 ml of PBS to the first tube containing the minced tissue. Cap the tube and, as in Step 3, invert it to resuspend the tissue pieces. Decant the supernatant into the second 50-ml tube containing pelleted cells, thus pooling all of the dissociated epithelial cells. (The minced tissue may now be discarded.) Pellet the pooled cells by centrifugation at 200*g* for 5 min at 20–25 °C. Discard the supernatant.

5| To ensure that dissociation enzymes are completely removed from the cells, resuspend the cell pellet in 20 ml of PBS, centrifuge again (200*g* for 5 min at 20–25 °C) and discard the supernatant.

6| Resuspend the cell pellet in 1–2 ml of FTM. To break up any cell clumps, gently pipette up and down at least 10 times using a 1-ml serological pipette.

7| Use a hemocytometer to count the number of secretory cells. There will be three types of cells in the cell suspension: ciliated cells (with beating cilia), secretory cells (nonciliated) and red blood cells (**Fig. 3a**). Count only the secretory cells. It is likely that there will be small clumps of epithelial cells (5–10 cells) that did not completely break up during Step 6. If you encounter this, try to estimate the number of cells in the clump as best you can.

? TROUBLESHOOTING

8| Dilute the cell suspension to 10^5 secretory cells per ml in FTM and plate 1 ml per well in a 24-well collagen-coated plate (**Fig. 3b**,**c**).

9| Incubate the cells overnight in a humidified 37 °C tissue culture incubator supplied with 5% CO_2.

10| Observe cells under the microscope. The secretory cells should adhere to the plate overnight and appear as nest-like 'islands' of cells (**Fig. 3d**). Ideally, cell confluency should be at least ~30%. However, because of the variable quality of fresh tissue samples, there may be some nonviable secretory cells, resulting in lower confluency. Ciliated cells often do not adhere to the plate, but if a ciliated cell does adhere (usually because it is still attached to a secretory cell) this is not a concern. Ciliated cells do not persist in 2D culture, although the reason for this is unclear. One possibility is that ciliated cells are terminally differentiated and therefore cannot proliferate. Another possibility is that ciliated cells undergo transdifferentiation to the secretory phenotype when cultured *in vitro*.

? TROUBLESHOOTING

11| Aspirate the medium from cells to remove blood cells and debris. Gently rinse the adherent cells with 500 µl of PBS using a serological pipette. If debris appears to be stuck on top of the cells, obscuring your view of them, do not rinse vigorously to remove it, as this may result in the detachment and loss of underlying secretory cells. Cell debris will usually detach on its own as the cells proliferate and can then be rinsed away. Aspirate the PBS and replace it with 500 µl of fresh FTM.

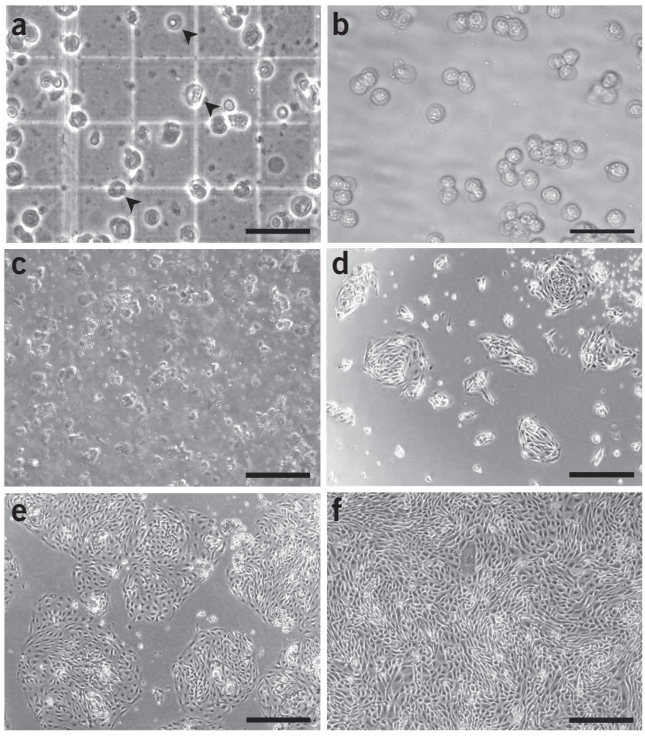

Figure 3 | Seeding and culture of dissociated FTSECs on collagen-coated plasticware. (**a**) Appearance of dissociated cells on a hemocytometer. The cell mixture obtained from enzymatic dissociation includes red blood cells (upper arrowhead), ciliated cells (middle arrowhead) and secretory cells (lower arrowhead). Ciliated cells are distinguished from secretory cells by the presence of cilia that beats very rapidly and is easily recognizable under the microscope. (**b**,**c**) Appearance of FTSECs immediately after plating, shown at ×10 (**b**) and ×4 (**c**) magnification, respectively. (**d**) One day after plating. Secretory cells attach to the plate and form 'islands'. The sample shown has been washed with PBS to remove unattached cells and debris. (**e**) Two days after plating. The cells are ready for transduction. (**f**) Three days after transduction with hTERT. The cells are 100% confluent and have a tight 'cobblestone' appearance. Reproduced with permission from Karst *et al.*[1]. Scale bars: **a**,**b**, 40 μm; **c**–**f**, 100 μm.

12| Continue to culture the cells until they reach ~50% confluency (**Fig. 3e**). This usually takes 1–2 d, depending on the sample. Change the medium every 24 h during this period to remove debris and prevent acidification of the medium.

▲ **CRITICAL STEP** If a sample grows rapidly and/or becomes 100% confluent before the cells can be transduced, split the sample at a ratio of 1:2 before transduction. To split cells, follow Steps 19–26. In Steps 25 and 26, however, plate cells in two wells of a 24-well plate instead of one well of a 12-well plate, and halve the volumes of medium used.

? TROUBLESHOOTING

Retroviral transduction with hTERT

13| When cells are ~50% confluent in a 24-well plate and ready for transduction (**Fig. 3e**), aspirate the medium and replace it with 1 ml of FTM supplemented with 8 μg ml^{-1} Polybrene. Polybrene is a cationic polymer used to increase the efficiency of retrovirus-mediated gene transfer.

14| Add sufficient hTERT retrovirus to achieve a dose of 2.5×10^5 infectious units per well. For example, if the viral titer is ~10^8 infectious units ml^{-1}, add 2.5 μl of virus per well. Swirl to mix.

15| Centrifuge the 24-well plate at 1,000*g* for 30 min at 37 °C.

16| Return cells to the incubator overnight.

17| Aspirate the retrovirus-containing medium and replace it with 500 μl of FTM.

18| Return cells to the incubator for a further 2 d.

▲ **CRITICAL STEP** We generally do not perform antibiotic selection for hTERT expression at this point because FTSECs expressing hTERT alone do not always grow well in our experience. Our immortalization success rates have been higher with hTERT-transduced cells, even when selection is not performed. If one wishes to eliminate hTERT-negative cells from the immortal cell population, it can easily be done at a later point in time, after the cells have achieved a more robust growth rate. To select an immortal FTSEC line for hTERT expression, culture the cells in FTM containing 200–400 μg ml^{-1} G418.

Retroviral transduction with p53 shRNA and $CDK4^{R24C}$

19| At 72 h post infection, FTSEC cultures should be 100% confluent (**Fig. 3f**). The cells must therefore be replated in larger wells to enable proliferation during the next round of retroviral transduction. To do this, aspirate the medium and rinse the cells with 500 μl of PBS.

? TROUBLESHOOTING

20| Aspirate the PBS and replace it with 250 μl of prewarmed trypsin.

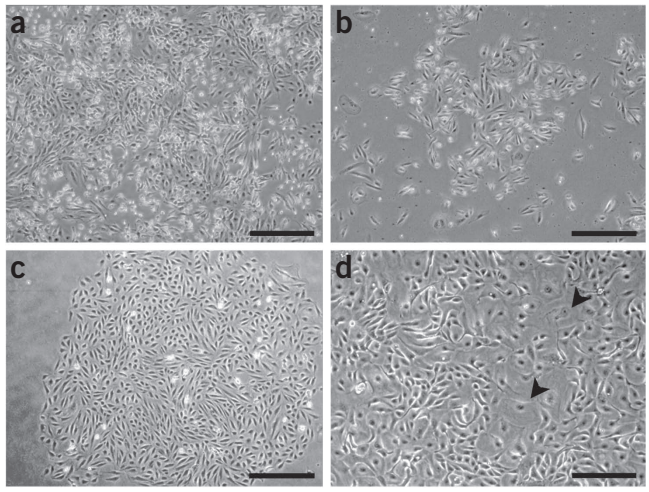

Figure 4 | Emergence of FTSECs colonies under selective pressure. (**a**) FTSECs transduced with neo-hTERT, puro-p53 shRNA and hygro-CDK4^{R24C} under puromycin selection for p53 shRNA expression. The cells have been exposed to selective medium for 2 d. Dying cells, which appear bright white, represent cells that were not successfully transduced with puro-p53 shRNA. (**b**) Seven days after the addition of selective medium. A puromycin-resistant colony of immortal cells slowly begins to emerge. (**c**) An expanding colony of immortal FTSECs. (**d**) Senescent FTSECs, which appear as large flattened cells (arrowheads), are commonly seen in primary and immortal cultures, even after antibiotic selection. Scale bars: **a**–**c**, 100 μm; **d**, 40 μm.

21| Place the plate of cells in the incubator and wait until the cells detach. This may take up to 10 min. Watch the cells closely to avoid overtrypsinization.
? TROUBLESHOOTING

22| When the cells have detached, add 500 μl of TNS to neutralize the trypsin. Pipette up and down to completely dislodge the cells from the plate.

23| Transfer all of the cells into one 15-ml conical tube. Rinse each well with 500 μl of PBS to collect residual cells and add them to the 15-ml tube as well.

24| Pellet the cells by centrifugation at 200*g* for 5 min at 20–25 °C. Discard the supernatant.

25| Resuspend the cell pellet in 1 ml of FTM for each trypsinized well. Plate the cells in a 12-well collagen-coated plate, adding 1 ml of cell suspension per well.

26| Collect residual cells by rinsing the tube with FTM (1 ml for each trypsinized well) and pipetting 1 ml into each well so that each well now contains 2 ml of cell suspension.

27| Add 16 μl of Polybrene solution to each well (final concentration = 8 μg ml^{-1}).

28| Add sufficient p53 shRNA virus to achieve a dose of 2.5 × 10^5 infectious units per well. Repeat with the CDK4^{R24C} virus. Swirl to mix. Each well should now contain a total retroviral dose of 5.0 × 10^5 infectious units.

29| Centrifuge the 12-well plate at 1,100*g* for 30 min at 37 °C.

30| Return the cells to the incubator overnight.

31| Aspirate the retrovirus-containing medium and replace it with 1 ml of FTM.

32| Return the cells to the incubator for a further 2 d.

Antibiotic selection of transduced cells

33| At 72 h after infection, aspirate the medium from the cells and replace it with 1 ml of FTM containing 0.5 μg ml^{-1} puromycin to select for cells expressing p53 shRNA.

34| Culture the cells for 1 week in puromycin-containing medium, replacing it every 2 d (**Fig. 4a**). If the cells become overconfluent, they may be split at a ratio of 1:2 during this time, as described in Steps 19–26. If the cells are subconfluent, do not split them.

Long-term culture

35| After 1 week of selection, when cell death has subsided, remove the puromycin from the cell culture medium. Continue to regularly check the cells. In approximately 2–4 weeks, small colonies of immortal cells should emerge and give rise to tightly packed colonies (**Fig. 4b**,**c**). Keep culturing the cells until the immortal colonies become confluent. A small proportion of surviving cells (~10%) may show a senescent morphology (**Fig. 4d**). This commonly occurs in early immortal FTSEC cultures.
? TROUBLESHOOTING

36| Trypsinize the immortal cells and split them at a ratio of 1:2. Representative examples of cell morphology after splitting are shown in **Figure 5**. As the cell population expands, trypsinize and replate the cells in larger wells, moving from 12-well plates, to six-well plates, to 100-mm plates during the expansion phase. Always split the cells at a ratio of 1:2–1:3. Once you reach the 100-mm plates, it is no longer necessary to coat the plates with collagen, nor is it necessary to use TNS. Instead, collect the trypsinized cells in FTM, pellet them, rinse with PBS, pellet the cells again and resuspend them in FTM for plating.

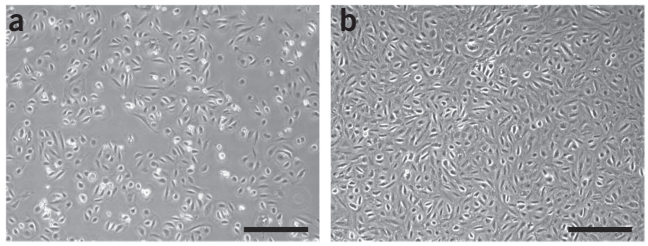

Figure 5 | Expansion of immortal FTSECs. (**a**) Immortal FTSECs after their first passage from 24-well to 12-well plates. (**b**) A confluent layer of immortal FTSECs. The cells show contact inhibition and can be cultured without splitting for a few weeks at a time. Scale bars, 100 μm.

37| When the cells have been expanded to two 100-mm plates, you may wish to culture them in hygromycin-containing medium (200–400 μg ml^{-1}) for 1 week to eliminate any cells that do not express $CDK4^{R24C}$. It is prudent to do this with only one plate at a time, in case the cells do not highly express $CDK4^{R24C}$ and a substantial amount of cell death occurs. Once selection is complete, it is not necessary to maintain the cells in selective medium.

Freezing immortal FTSECs

38| Once immortal FTSECs have been expanded to three or more 100-mm plates, some cells should be frozen down and stored in liquid nitrogen to preserve a low-passage population. First, trypsinize the cells, collect them in FTM and pellet them by centrifugation at 200*g* for 5 min at 20–25 °C.

39| Discard the supernatant and resuspend pelleted cells in 2–3 ml of PBS to rinse away residual trypsin. Re-pellet the cells by centrifugation at 200*g* for 5 min at 20–25 °C.

40| Discard the supernatant and resuspend the cells in FTM containing 10% (vol/vol) DMSO at a concentration of 10^6 cells ml^{-1}.

41| Aliquot 1 ml each into cryogenic vials and store them at −80 °C overnight.

42| The next day, transfer the vials to a liquid nitrogen storage tank. Early-passage immortal cells (up to p10) tolerate freezing fairly well and typically maintain ≥70% viability upon thawing. Later passages (p10–p30) are more tolerant and show very little cell death upon thawing.

■ **PAUSE POINT** Immortal FTSEC lines may be stored in liquid nitrogen for at least 5 years.

Thawing immortal FTSECs

43| To thaw cells, remove a vial from liquid nitrogen and place it immediately into a 37 °C water bath, taking care not to immerse the cap.

! CAUTION Wear a protective face shield when removing vials from liquid nitrogen. Improperly sealed vials containing liquid nitrogen can explode owing to rapid expansion at room temperature.

44| Once the ice crystals melt, promptly remove the vial from the water bath and use a pipette to transfer the contents to a 10-cm tissue culture dish containing 9 ml of prewarmed FTM.

45| Change the medium within 24 h to minimize exposure of cells to DMSO and remove dead cells.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7	Low cell number	Insufficient mincing of tissue, resulting in suboptimal enzyme access	Chop the tissue into very small pieces, <1 mm^3
		Tissue sample was too small	Repeat the protocol with a larger sample
10	No cells adhered to the plate	Cells in the tissue sample were not viable	Poor sample quality. Nonmotile cilia (Step 7) may indicate poor sample quality. Repeat the protocol with a new sample
		Tissue sample was left sitting out too long before it was immersed in PBS	Minimize the time that the tissue is exposed to air during sample collection
		Too much time elapsed between tissue collection and tissue processing	Process the tissue (Steps 1 and 2) as soon as possible after receiving the sample
12	Cells stop growing before reaching 50% confluency	Cells in the tissue sample were not proliferative enough	Cell proliferation rates vary considerably with each sample and are sometimes insufficient for *in vitro* culture. Repeat the protocol with a new sample
		Cells were seeded too sparsely in Step 8	Increase the number of cells per well when plating cells
19	Cells appear static and do not grow to 100% confluency	Virus titer was too high and cells underwent arrest	Decrease the amount of virus added to cells in Step 14
21	Cells will not detach during trypsinization	The cells have formed very strong attachments to the collagen	Extend trypsinization time until ~50% of the cells lift off the plate. Gently pipette the trypsin up and down using with a 1,000-µl pipette to promote cell detachment. Transfer the cells to a clean tube containing 500 µl of TNS. Add 250 µl of additional trypsin to the well and repeat this process. It may take a few trypsinization-neutralization cycles to collect all of the cells. If a few stubborn cells refuse to detach, you can simply leave them behind and proceed with the protocol, or you can try transducing them *in situ*, in parallel with the detached cells. This will increase the total amount of infection medium and virus needed for Steps 13 and 14
35	No colonies have emerged after 2–3 weeks	Inadequate transduction efficiency	Increase the viral dose in Step 28
		Immortal cells grow very slowly	In some cases it takes several weeks for colonies to emerge. Therefore, it is best not to discard the cells prematurely. Keep the cells in culture, changing the medium every 2–3 d and wait for colonies to appear. In the meantime, repeat the protocol with a new sample

● TIMING

Steps 1 and 2 (day 1): collect fallopian tube sample (REAGENT SETUP) and begin epithelial dissociation
Day 2: no action required
Steps 3–9 (day 3): quantify and plate dissociated FTSECs
Steps 10–12 (days 4 and 5): maintain primary FTSEC cultures
Steps 13–16 (day 6): transduce primary cultures with hTERT retrovirus
Steps 17 and 18 (day 7): replace culture medium
Day 8: no action required

Steps 19–30 (day 9): transduce cultures with p53 shRNA and CDK4^{R24C} retroviruses
Steps 31 and 32 (day 10): replace culture medium
Day 11: no action required
Step 33 (day 12): begin antibiotic selection of transduced FTSECs
Step 34 (days 13–18): continue antibiotic selection
Steps 35–37 (next 2–4 weeks): culture surviving cells and monitor cultures for immortal colony growth
Steps 38–41 (as required): freezing
Steps 42–45 (as required): thawing immortal FTSEC lines

ANTICIPATED RESULTS

Before using immortal FTSECs for experiments, a western blot analysis should be performed to verify that immortal cells maintain the Müllerian phenotype, which is a fundamental characteristic of FTSECs. Expression of PAX8, a Müllerian lineage marker[1,13,26,27], and CK7, a specific cytokeratin expressed by FTSECs, indicates that cells have not dedifferentiated in culture (**Fig. 6a**). Western blotting can also be used to validate the presence of genetic elements introduced into the cells (**Fig. 6a**). Cell lysates for western blotting may be prepared using any standard cell lysis buffer. To detect hTERT expression, which can sometimes be problematic by western blotting, RT-PCR can alternatively be used, as previously described[1]. In addition to validating the presence of a genetic alteration, it may be useful to assess its functional activity. For example, CDK4^{R24C} activity should induce phosphorylation of pRb at Ser780, which is a specific CDK4 target in pRb pathway activation (**Fig. 6b**). Similarly, telomerase activity resulting from hTERT expression may be measured using the telomeric repeat amplification protocol[28]. Last, it is a good idea to perform immunofluorescent staining for PAX8 (**Fig. 6c**) in order to detect any PAX8-negative cells. Large numbers of PAX8-negative cells may indicate that a contaminating cell type (for example, fibroblasts) may have been inadvertently immortalized along with FTSECs. Conditions for western blotting and immunofluorescent staining of FTSECs (including antibody specifications) have been previously described[1].

Because multiple antibiotic resistance markers are used to generate immortal cell lines, it is not possible to keep immortal FTSECs under long-term selective pressure for all of the genetic alterations introduced. An immortal FTSEC line will normally maintain high-level expression of any genetic alteration on which it depends for viability or that confers a significant growth advantage. However, other genetic elements may not be crucially required after immortalization, and consequently their expression may be lost at some point in the absence of selective pressure. In our experience, immortal FTSECs typically maintain p53 perturbations (such as p53 shRNA, mutant p53 or SV40 large T antigen), whereas CDK4^{R24C} levels sometimes decrease over time. In this context, it is possible that CDK4^{R24C} has a greater role in the immortalization process than in sustaining FTSEC proliferation.

Most immortal FTSEC lines can be passaged continuously for at least 1 year. However, their growth rate and cell morphology will likely change over time. Therefore, we recommend using low-passage cells whenever possible. It is a good idea to periodically perform western blot analysis of immortal cell lines to ensure that the cells still express PAX8, CK7 and other relevant markers. In addition, long-term culture of immortal cells may eventually lead to the acquisition of genetic defects, either spontaneously or as a direct consequence of the loss of p53 and pRb tumor suppressor function. Immortal cells should not show anchorage-independent growth in soft agar.

If cells acquire the ability to grow in soft agar, they have likely undergone a substantial genomic change. To assess the genomic integrity of an immortal FTSEC line, we recommend that array comparative genomic hybridization (aCGH) analysis be performed.

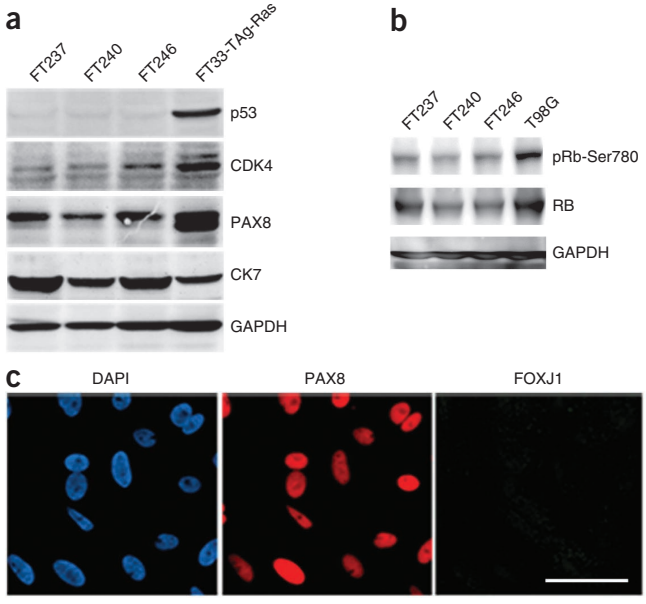

Figure 6 | Validation of immortal FTSECs. (**a**) Western blots of immortal FTSEC lines derived from three different patients (FT237, FT240 and FT246). The cells express PAX8 and CK7, indicating that they maintain a Müllerian phenotype *in vitro*. They also express the genetic alterations used to immortalize them; namely, p53 knockdown and CDK4^{R24C}. The positive control (FT33-Tag-Ras) is a Ras-transformed FTSEC line, previously described[1]. Antibody dilution: p53, CDK4, PAX8 and CK7, 1:1,000; GAPDH, 1:2,000. (**b**) CDK4^{R24C} kinase activity results in hyperphosphorylation of pRb at Ser780. The positive control (T98G) is a glioblastoma cell line. Antibody dilution: pRb-Ser780 and RB, 1:1,000; GAPDH, 1:2,000. (**c**) Immunofluorescent staining of immortal FTSECs for PAX8 confirms that all cells are of the secretory cell type. They are negative for FOXJ1, a marker of FTE ciliated cells DAPI staining identifies the cell nuclei. Reproduced with permission from Karst *et al.*[1]. Antibody dilution: PAX8, 1,000; FOXJ1, 1:750. Scale bar, 30 μm.

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AUTHOR CONTRIBUTIONS A.M.K. developed the experimental protocol, performed the experiments and wrote the manuscript. R.D. contributed to the conceptual design of the protocol and edited the manuscript.

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1. Karst, A.M., Levanon, K. & Drapkin, R. Modeling high-grade serous ovarian carcinogenesis from the fallopian tube. *Proc. Natl. Acad. Sci. USA* **108**, 7547–7552 (2011).
2. Medeiros, F. *et al.* The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome. *Am. J. Surg. Pathol.* **30**, 230–236 (2006).
3. Lee, Y. *et al.* A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *J. Pathol.* **211**, 26–35 (2007).
4. Crum, C.P. Intercepting pelvic cancer in the distal fallopian tube: theories and realities. *Mol. Oncol.* **3**, 165–170 (2009).
5. Przybycin, C.G., Kurman, R.J., Ronnett, B.M., Shih Ie, M. & Vang, R. Are all pelvic (nonuterine) serous carcinomas of tubal origin? *Am. J. Surg. Pathol.* **34**, 1407–1416 (2010).
6. Crum, C.P. *et al.* The distal fallopian tube: a new model for pelvic serous carcinogenesis. *Curr. Opin. Obstet. Gynecol.* **19**, 3–9 (2007).
7. Kurman, R.J. & Shih Ie, M. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer—shifting the paradigm. *Hum. Pathol.* **42**, 918–931 (2011).
8. Salvador, S. *et al.* The fallopian tube: primary site of most pelvic high-grade serous carcinomas. *Int. J. Gynecol. Cancer* **19**, 58–64 (2009).
9. Karst, A.M. & Drapkin, R. Ovarian cancer pathogenesis: a model in evolution. *J. Oncol.* **2010**, 932371 (2010).
10. Levanon, K., Crum, C. & Drapkin, R. New insights into the pathogenesis of serous ovarian cancer and its clinical impact. *J. Clin. Oncol.* **26**, 5284–5293 (2008).
11. Greene, M.H., Mai, P.L. & Schwartz, P.E. Does bilateral salpingectomy with ovarian retention warrant consideration as a temporary bridge to risk-reducing bilateral oophorectomy in *BRCA1/2* mutation carriers? *Am. J. Obstet. Gynecol.* **204**, 19.e1–19.e6 (2011).
12. Folkins, A.K., Jarboe, E.A., Roh, M.H. & Crum, C.P. Precursors to pelvic serous carcinoma and their clinical implications. *Gynecol. Oncol.* **113**, 391–396 (2009).
13. Levanon, K. *et al.* Primary *ex vivo* cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. *Oncogene* **29**, 1103–1113 (2010).
14. Fotheringham, S., Levanon, K. & Drapkin, R. *Ex vivo* culture of primary human fallopian tube epithelial cells. *J. Vis. Exp.* doi:10.3791/2728 (2011).
15. Karst, A.M. & Drapkin, R. The new face of ovarian cancer modeling: better prospects for detection and treatment. *F1000 Med. Rep.* **3**, 22 (2011).
16. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature* **474**, 609–615 (2011).
17. Pearce, C.L. *et al.* Validating genetic risk associations for ovarian cancer through the International Ovarian Cancer Association Consortium. *Br. J. Cancer* **100**, 412–420 (2009).
18. Fasching, P.A. *et al.* Role of genetic polymorphisms and ovarian cancer susceptibility. *Mol. Oncol.* **3**, 171–181 (2009).
19. Goode, E.L. *et al.* A genome-wide association study identifies susceptibility loci for ovarian cancer at 2q31 and 8q24. *Nat. Genet.* **42**, 874–879 (2010).
20. Etemadmoghadam, D. *et al.* Integrated genome-wide DNA copy number and expression analysis identifies distinct mechanisms of primary chemoresistance in ovarian carcinomas. *Clin. Cancer Res.* **15**, 1417–1427 (2009).
21. Notaridou, M. *et al.* Common alleles in candidate susceptibility genes associated with risk and development of epithelial ovarian cancer. *Int. J. Cancer* **128**, 2063–2074 (2011).
22. Bolton, K.L. *et al.* Common variants at 19p13 are associated with susceptibility to ovarian cancer. *Nat. Genet.* **42**, 880–884 (2010).
23. Jazaeri, A.A. *et al.* Molecular requirements for transformation of fallopian tube epithelial cells into serous carcinoma. *Neoplasia* **13**, 899–911 (2011).
24. Miller, D.G., Adam, M.A. & Miller, A.D. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell Biol.* **10**, 4239–4242 (1990).
25. Kanbe, E. & Zhang, D.E. A simple and quick method to concentrate MSCV retrovirus. *Blood Cells Mol. Dis.* **33**, 64–67 (2004).
26. Laury, A.R. *et al.* PAX8 reliably distinguishes ovarian serous tumors from malignant mesothelioma. *Am. J. Surg. Pathol.* **34**, 627–635 (2010).
27. Bowen, N.J. *et al.* Emerging roles for PAX8 in ovarian cancer and endosalpingeal development. *Gynecol. Oncol.* **104**, 331–337 (2007).
28. Herbert, B.S., Hochreiter, A.E., Wright, W.E. & Shay, J.W. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. *Nat. Protoc.* **1**, 1583–1590 (2006).