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Reconstruction of Oviduct and Demonstration of Epithelial Fate Determination in Mice[1]

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ABSTRACT

The mouse oviductal epithelium is a simple monolayer until Postnatal Day 7 and subsequently consists of differentiated secretory cells and ciliated cells. In adult oviduct, the two types of epithelial cells are unevenly distributed; ciliated cells are dominant in the ampulla and secretory cells are dominant in the isthmus. Recombinants of enzymatically separated epithelial and mesenchymal tissues of oviducts were grafted under kidney capsule for 4 wk. The recombinants developed structures with a lumen covered with a monolayer of ciliated cells and secretory cells, demonstrating that the recombinant tissues reconstructed oviductal structure. Geographically (ampulla versus isthmus) heterotypic recombinants were prepared from neonatal oviducts at Day 3. The epithelia in reconstructed oviducts took the patterns of cell distribution depending on the origin of the mesenchymal tissues. The results indicate that the mesenchyme geographically has distinct abilities to determine undifferentiated epithelial cells to ciliated cells or secretory cells in the mouse oviduct.

epithelial-mesenchymal interaction, fallopian tubes, female reproductive tract, Müllerian ducts, oviduct

INTRODUCTION

The Müllerian duct develops from the intermediate mesoderm and gives rise to the oviduct, uterus, and upper portion of vagina. In female mouse embryo, the Müllerian duct develops in parallel to the Wolffian duct around E11.5 (vaginal plug = E0.5) and reaches the cloaca by E13.5 [1]. The Wolffian duct begins to degenerate around E15, and two horns of the Müllerian duct fuse around E15.5. Around E16, Müllerian vaginal epithelium and endoderm-derived sinus vaginal epithelium fuse. At E18, simple columnar epithelial cells are found in the oviduct, uterus, and Müllerian vagina [2]. During postnatal development, the epithelia of Müllerian duct-derived organs undergo specific morphogenetic changes.

In adult mouse, the oviductal epithelium consists of two major cell populations, secretory cells and ciliated cells [3]. The uterine epithelium is composed of simple columnar and glandular cells, whereas the vaginal epithelium is stratified-squamous. The oviduct is a simple tubular structure in embryos and morphologically develops four compartments in adults: infundibulum (Inf), ampulla (Amp), isthmus (Ist), and uterotubal junction [4]. The epithelium morphologically changes from undifferentiated columnar cells to ciliated cells and secretory cells. More ciliated cells than secretory cells are observed in Inf/Amp. By contrast, more secretory cells than ciliated cells are present in Ist. The cause of the regionally different distribution of the two types of cells is not known.

Tissue recombinant experiments demonstrated that the fate of epithelial cells in the uterus and Müllerian vagina is determined by the reciprocal interactions between the epithelium and the underlying mesenchyme [5–7]. Until Postnatal Day 7 (P7), uterine epithelium can differentiate into vaginal epithelium when combined with vaginal mesenchyme, while vaginal epithelium differentiates into uterine epithelium when combined with uterine mesenchyme. The plasticity of the undifferentiated epithelia is gradually lost over the long period of time [2, 5]. Thus, the fate of epithelial cells in uterus and vagina is determined by the underlying mesenchyme.

Due to its small structure, however, little is known about the development of the mouse oviduct. Additionally, no experimental methods have been established to elucidate the developmental mechanism of the oviduct. In the present study, we succeeded in adapting the tissue recombinant method for study of oviductal development, and the method has clearly demonstrated that the mesenchyme has geographically distinct abilities to determine the undifferentiated epithelium to cilial type or secretory type.

MATERIALS AND METHODS

Animals

CD1 mice (Charles River Japan, Yokohama, Japan) and GFP mice (C57BL/6-*Tg (CAG-EGFP)*) (Japan SLC, Inc., Shizuoka, Japan) were maintained in the experimental animal facility of Tokyo University of Science. They were kept under a 12L:12D cycle at 22–24°C. Standard laboratory feed (MR standard; Nousan LTD, Yokohama, Japan) and tap water were given ad libitum. Detection of a vaginal plug at noon was designated as embryonic day 0.5 (E0.5), and the day of birth was designated as P0. The stage of estrus cycle was judged by vaginal smear. Mice care and handling conformed to the guidelines for animal research of National Institutes of Health. The Institutional Animal Care and Use Committee approved the experimental protocols.

Preparation of Tissue Recombinants and Grafting under the Kidney Capsule

Oviducts were dissected from CD1 and GFP mice on P3. They were cut into two pieces (Inf/Amp and Ist), placed into 1% trypsin (Invitrogen, Carlsbad, CA) in Hanks balanced salt solution (HBSS) (Sigma, St. Louis, MO), and incubated at 4°C for 90 min. The pieces of tissues were washed with 20% fetal calf serum (FCS) in HBSS and treated with deoxyribonuclease 1-A (Sigma). Epithelium and mesenchyme were separated by gently sucking them into a capillary under a stereomicroscope. Histological examination confirmed that separated tissues were not contaminated with other tissues. For recombinant

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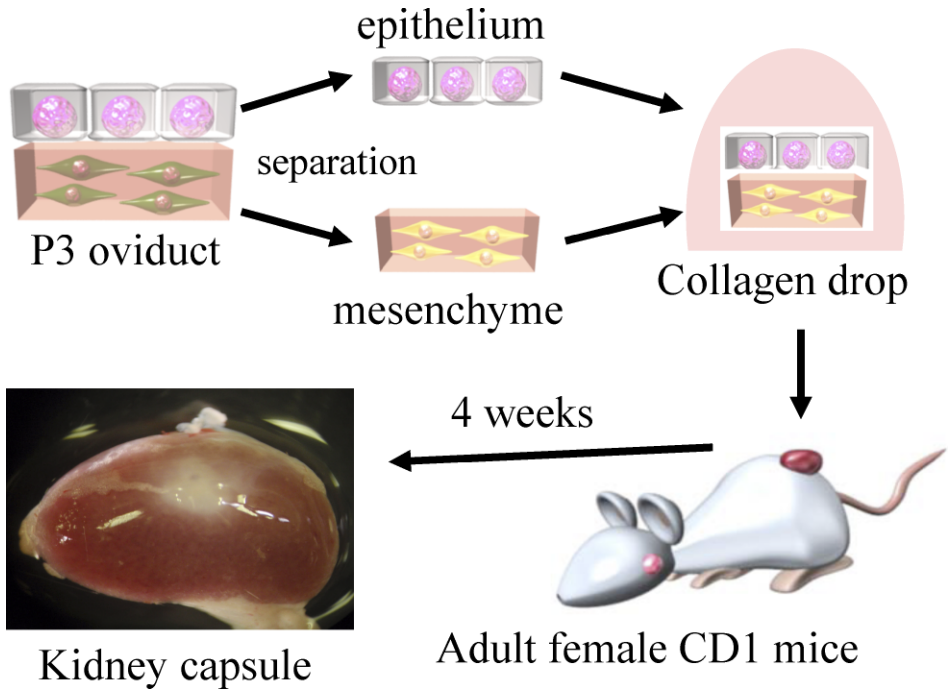

FIG. 1. Schematic drawing of tissue recombinant method. Epithelial and mesenchymal tissues were separated with trypsin. Separated tissues were recombined in a collagen drop. The recombinants were grafted under a kidney capsule (Magnification of stereoscopic microscope ×4) of adult female mice for 4 wk.

preparation, a mesenchymal tissue was put into a 20-μl gel drop of Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan) on a siliconized dish. Epithelial tissue was injected into an adjacent area of mesenchymal tissue in the matrix using a superfine tip. A collagen drop containing a recombinant was incubated for 5 min at 37°C, placed on a cell culture insert, and cultured for 1 day in 10% FCS at 37°C in a humidified atmosphere of 5% CO_2. After the incubation, four recombinants per kidney were bilaterally grafted under the kidney capsule of an adult female CD1 mouse. Kidneys were harvested and processed for histological and immunohistochemical staining 4 wk after grafting. The procedure described above is schematically drawn in Figure 1.

Histological and Immunohistochemical Analyses

Harvested kidneys and oviductal tissues were fixed overnight in 4% formaldehyde at 4°C and dehydrated with graded alcohol. They were embedded in paraffin and cut into 6-μm sections. Sections were deparaffinized with xylene, and rehydrated with graded ethanol. Then they were stained with hematoxylin and eosin. For immunohistochemical staining, sections were deparaffinized and rehydrated with graded ethanol. The sections were washed twice in phosphate-buffered saline (PBS) with 0.05% Tween 20, and nonspecific binding was blocked by incubation for 1 hr at room temperature (RT) in PBS containing 1% bovine serum albumin (Trace Biosciences Pty Ltd., New South Wales, Australia), 5% normal goat serum (Zymed Laboratories Inc., San Francisco, CA), and 0.4% Triton X-100 (Sigma). The sections were then incubated with primary antibodies overnight at 4°C. The primary antibodies were anti-OVGP1 polyclonal antibody (oviductin, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-tubulin IV monoclonal antibody (tubb4, 1:100; Boehringer Mannheim Biochemica, Mannheim, Germany). After washing three times with cold PBS, the slides were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG serum (Biosource International, Camarillo, CA) or Cy3-conjugated anti-rabbit IgG serum (Biosource International) at RT for 2 hr. 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was used for counter staining. Negative controls were incubated without primary antibodies. Samples were observed with a fluorescence microscope (Carl Zeiss, Oberkochem, Germany).

The 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, Jena, Germany) interfaced with an Axiovert 200M (Carl Zeiss). Results were based on analysis of 8–24 tissue recombinants per group. Data were analyzed by Student *t* test or ANOVA test. A statistically significant difference was defined as $P < 0.05$.

RESULTS

The Oviductal Epithelium Consists of Secretory Cells and Ciliated Cells

The epithelium of an adult oviduct consists of two major populations, secretory cells and ciliated cells. To investigate the development and distribution of the two types of epithelial cells, immunohistochemical analysis using anti-β-tubulin IV and anti-OVGP1 antibodies was performed. Beta-tubulin IV is an essential component of cilia, and OVGP1 is one of secretory glycoproteins of mouse oviduct [8]. In P3 oviduct, no β-tubulin IV-positive cells were detected, and OVGP1-positive cells were seen in the epithelium of Amp (Fig. 2, A–C). In adult oviduct, the epithelium was occupied mainly by ciliated cells in Inf and Amp (Fig. 2, D and E). Beyond the border between Amp and Ist, OVGP1-positive cells were mainly observed in the epithelium (Fig. 2, E and F). These results demonstrated that many undetermined (negative for both β-tubulin IV and OVGP1) epithelial cells exist in oviduct at P3. Thereafter, they differentiate into ciliated or secretory cells. Cells double positive for β-tubulin IV and OVGP1 were not observed at any of the stages examined.

The Ratio of the Two Types of Epithelial Cells Remains Unchanged in Adult Oviduct

At estrus, the epithelium in Amp had ciliated cells at 79.0% ± 0.2% and secretory cells at 21.0% ± 0.2% (n = 3). The epithelium in Ist had ciliated cells at 11.5% ± 3.2% and secretory cells at 88.5% ± 3.2% (n = 3) (Fig. 2G). At diestrus, the epithelium in Amp had ciliated cells at 77.9% ± 2.9% and secretory cells at 22.1% ± 2.9% (n = 3). The epithelium in Ist had ciliated cells at 6.8% ± 7.1% and secretory cells at 93.2% ± 7.1% (n = 3) (Fig. 2G). The distribution pattern of the two cell populations was not significantly different between the two stages of the estrus cycle ($P > 0.05$). In adults, ciliated cells are a major population in Amp epithelium, and secretory cells are a major population in Ist epithelium.

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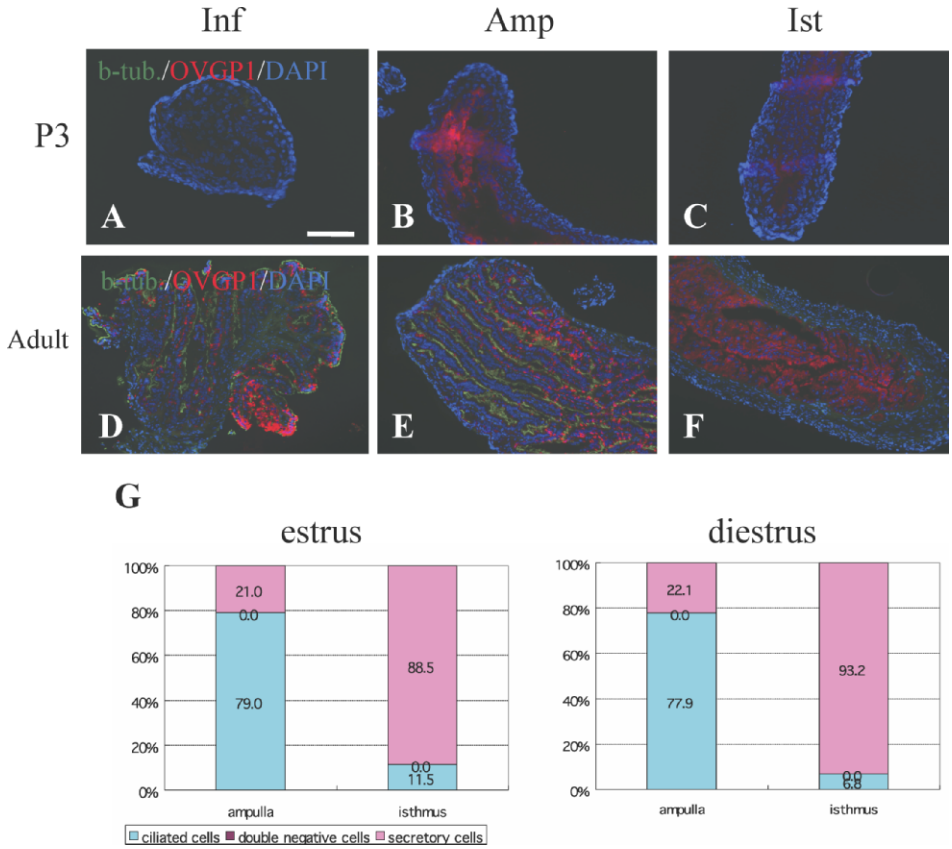

FIG. 2. Distribution pattern of β-tubulin IV-positive cells and OVGP1-positive cells in the oviduct. Double immunolabeling for β-tubulin IV (green) and OVGP1 (red) in oviducts. Sections were counterstained with DAPI (blue). Inf, Infundibulum; Amp, ampulla; Ist, isthmus. Bars = 100 μm. **A**) Neither expression of β-tubulin IV nor OVGP1 were detected in the epithelium of Inf at P3. **B**) A few β-tubulin IV-positive cells and many OVGP1-positive cells were observed in the epithelium of Amp at P3. **C**) Neither β-tubulin IV-positive cells nor OVGP1-positive cells were detected in the epithelium of Ist at P3. **D**) Beta-tubulin IV-positive cells and OVGP1-positive cells were recognized in the epithelium of adult Inf. Beta-tubulin IV-positive cells were dominant. **E**) Beta-tubulin IV-positive cells and OVGP1-positive cells were distributed in the epithelium of adult Amp as seen in Inf. **F**) The epithelium of adult Ist was occupied by mainly OVGP1-positive cells (in the epithelium of adult Ist). **G**) Numbers of ciliated cells and secretory cells were counted in adult Amp and Ist at estrus and diestrus, and the ratios were calculated. At estrus, epithelia of Amp (AmpE) consisted of ciliated cells at 79.0% ± 0.2% and secretory cells at 21.0% ± 0.2%. However, epithelia of Ist had ciliated cells at 11.5% ± 3.2% and secretory cells at 88.5% ± 3.2%. At diestrus, epithelia of Amp consisted of ciliated cells at 77.9% ± 2.9% and secretory cells at 22.1% ± 2.9%. Epithelia of Ist had ciliated cells at 6.8% ± 7.1% and secretory cells at 93.2% ± 7.1%.

Reconstruction of Oviductal Structure

Oviducts (P3) were enzymatically separated into epithelial and mesenchymal tissues and recombined and grafted under the kidney capsule. The grafts developed structures with a lumen surrounded by a cell layer of ciliated or nonciliated cells (Fig. 3, A and F). Immunohistochemical analyses demonstrated that the epithelium had β-tubulin IV-positive (ciliated) cells or OVGP1-positive (secretory) cells when combined and grafted under the kidney capsule for 4 wk (Fig. 3, C and H). The results demonstrated that tissue recombinants reconstructed oviductal structure, and epithelial cells differentiated into ciliated cells or secretory cells during the grafting period.

Tissue Recombinants Demonstrate That the Mesenchyme Determines the Fate of Epithelial Cells

As observed in the preceding experiments, two types of epithelial cells are unevenly distributed in adult oviduct. The epithelial cell fate is determined by the underlying mesenchyme in vagina and uterus during a neonatal period [2, 7], suggesting that the uneven distribution of epithelial cells in oviduct is also caused by the geographically localized mesenchymal cells at Amp and Ist. To examine the possibility, geographically homotypic and heterotypic recombinants between epithelium and mesenchyme were prepared and grafted under the kidney capsule. When combined with mesenchyme of Amp at P3 (P3-AmpM), epithelium of Amp at P3 (P3-AmpE) differentiated into β-tubulin IV-positive ciliated cells at 73.6% ± 9%, OVGP1-positive secretory cells at 13.2% ± 3.8%, and double-negative cells at 13.2% ± 8.4% (n = 12) (Figs. 3, A and C, and 4). When combined with P3-AmpM, epithelium of Ist at P3 (P3-IstE) differentiated into β-tubulin IV-positive ciliated cells at 67.2% ± 12.8%, OVGP1-positive secretory cells at 19.0% ± 6.6%, and double-negative cells at 13.8% ± 7.5% (n = 8) (Figs. 3, B and D, and 4). When combined with P3-IstM, P3-IstE differentiated into β-tubulin IV-positive ciliated cells at 17.6 ± 7.5%, OVGP1-positive secretory cells at 70.3% ± 1.8%, and double-negative cells at 12.1% ± 3.0% (n = 8) (Figs. 3, F and H, and 4). When combined with P3-IstM (n = 12), P3-AmpE differentiated into β-tubulin IV-positive ciliated cells at 13.5% ± 4.5%, OVGP1-positive secretory cells at 74.3% ± 6.9%, and double-negative cells at 12.2% ± 3.0% (n = 12) (Figs. 3, E and G, and 4). Thus, geographically homotypic and heterotypic recombinants between epithelium and mesenchyme demonstrated that the local mesenchyme determines the distribution pattern of the two types of epithelial cells.

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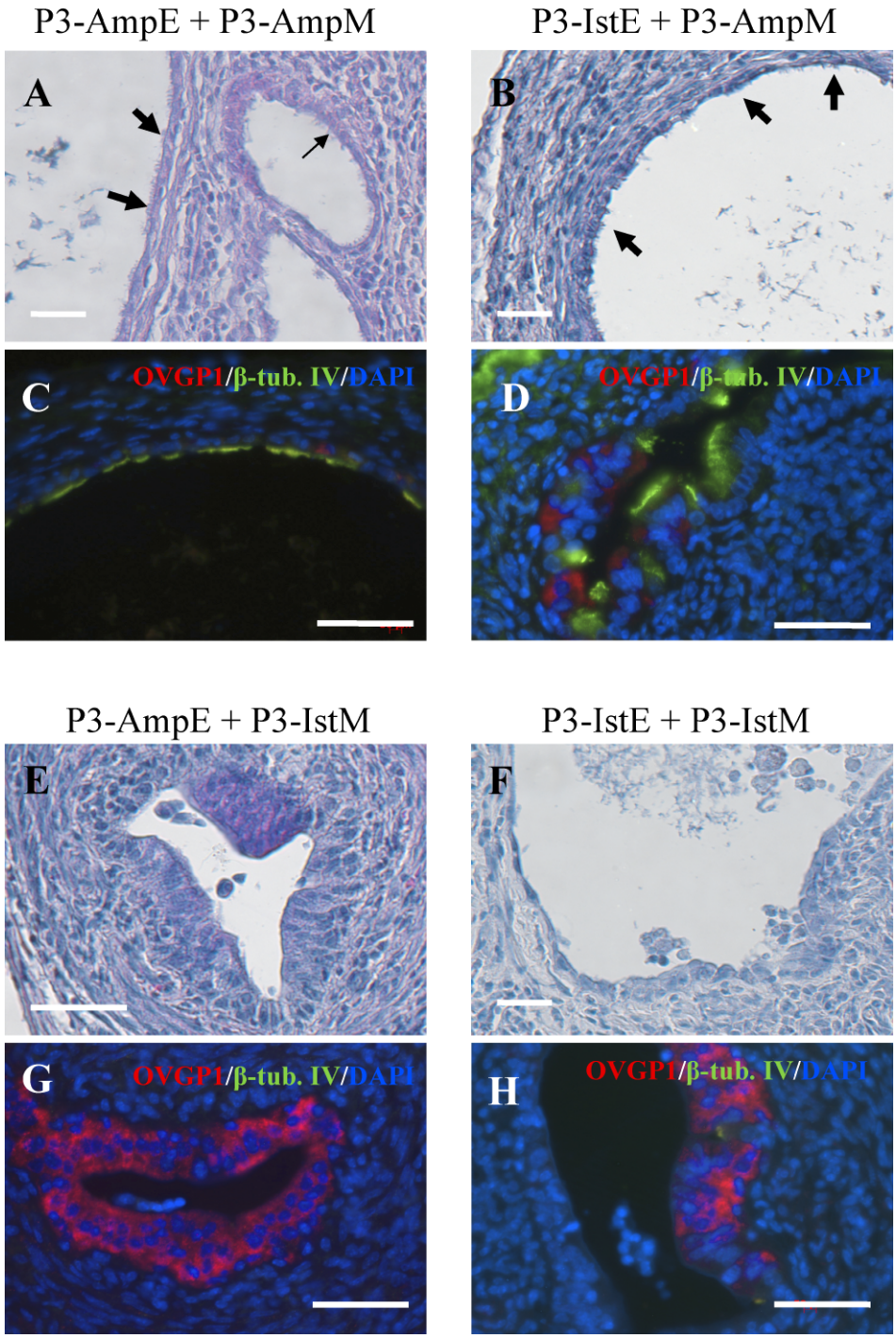

FIG. 3. Histology and immunohistochemistry of reconstructed oviducts. P3-AmpM (mesenchyme of ampulla at P3) were recombined with P3-AmpE (epithelium of ampulla at P3) or P3-IstE (epithelium of isthmus at P3) and grafted under kidney capsule for 4 wk. The recombinant tissues developed miniature oviducts. Immunostained sections were counterstained with DAPI (blue). Bars = 100 μm. **A**) P3-AmpE recombined with P3-AmpM developed an epithelium with numerous cilia (arrows). **B**) P3-IstE recombined with P3-AmpM developed an epithelium with numerous cilia (arrows). **C**) Double immunostaining for β-tubulin IV (green) and OVGP1 (red) of **A**. Beta-tubulin IV-positive cells occupied the epithelium. **D**) Double immunostaining for β-tubulin IV and OVGP1 of **B**. Beta-tubulin IV-positive cells occupied the epithelium. P3-IstM (mesenchyme of isthmus at P3) were recombined with P3-AmpE (epithelium of ampulla at P3) or P3-IstE (epithelium of isthmus at P3) and grafted under kidney capsule for 4 wk. **E**) P3-AmpE recombined with P3-IstM developed an epithelium of simple columnar cells without cilia. **F**) P3-AmpE recombined with P3-IstM developed an epithelium of simple columnar cells without cilia. **G**) Double immunostaining for β-tubulin IV and OVGP1 of **E**. OVGP1-positive cells occupied the epithelium. **H**) Double immunostaining for β-tubulin IV and OVGP1 of **F**. OVGP1-positive cells occupied the epithelium.

Single Graft of a Piece of Epithelium or Mesenchyme under Kidney Capsule

When a piece of epithelium (P3-AmpE) or mesenchyme (P3-AmpM) was singly grafted, cell clusters developed from the grafts, and none of them had oviductal structure (Fig. 5, A and B). Thus, any epithelial or mesenchymal tissue grafted singly under kidney capsule was unable to reconstruct oviductal structure, suggesting that separation of the epithelium and mesenchyme was completed. To further assess the possible contribution of residual epithelial cells, mesenchymal tissues prepared from oviducts of GFP mice were recombined with epithelial tissues prepared from oviducts of CD1 mice, and they were grafted under the kidney capsule. The grafts developed oviductal structures with epithelia containing no GFP-positive cells (data not shown).

Determined Epithelial Cells Do Not Have Plasticity

Until P7, epithelia separated from uterus and vagina can differentiate to uterine or vaginal epithelium when combined with either uterine or vaginal mesenchyme. Then, the plasticity of the epithelia is gradually lost [2]. To examine the plasticity of epithelium of adult oviduct, epithelia were prepared from adult Amp (adult-AmpE) and combined with neo-IstM and grafted under the kidney capsule. The reconstructed epithelia had the same pattern of cell populations as observed in adult-AmpE (Fig. 6A). Similarly, epithelia were prepared from adult-

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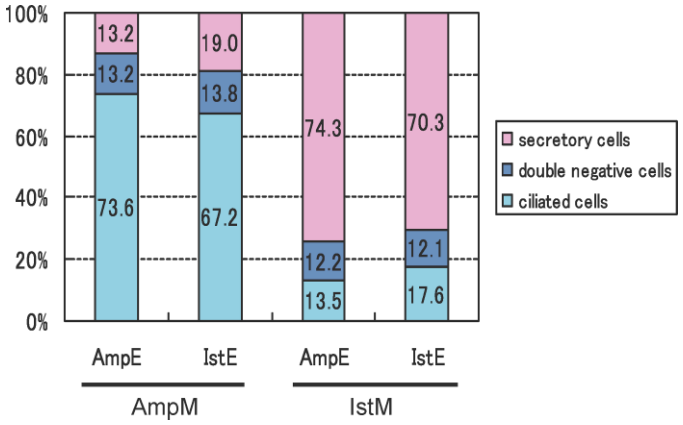

FIG. 4. Numbers of OVGP1-positive cells and β-tubulin IV-positive cells in reconstructed oviducts. Numbers of OVGP1-positive cells, β-tubulin IV-positive cells and nonlabeled cells were counted in reconstructed oviducts, and the ratios were calculated. In combination with P3-AmpM, P3-AmpE developed epithelia consisting of ciliated cells at 73.6% ± 9.0%, secretory cells at 13.2% ± 3.8%, and double-negative cells at 13.2% ± 8.4%. While P3-IstE developed epithelia consisting of ciliated cells at 67.2% ± 12.8%, secretory cells at 19.0% ± 6.6%, and double-negative cells at 13.8% ± 7.5%. In combination with P3-IstM, P3-AmpE developed epithelia consisting of ciliated cells at 13.5% ± 4.5%, secretory cells at 74.3% ± 6.9%, and double-negative cells at 12.2% ± 3.0%. P3-IstE developed epithelia consisting of ciliated cells at 17.6% ± 7.5%, secretory cells at 70.3% ± 1.8%, and double-negative cells at 12.1% ± 5.8%.

IstE and combined with neo-AmpM and grafted under the kidney capsule. The reconstructed epithelia had the same pattern of cell populations as observed in adult-IstE (Fig. 6B). These results indicate that the epithelial cells in adult oviducts have lost their plasticity.

DISCUSSION

Research of developmental mechanisms in female reproductive tracts has been mainly focused on the uterus and vagina, and developmental biology of the oviduct has been poorly understood. In the present study, we first observed the basic epithelial histoarchitecture of oviducts, and then we established experimental model systems in which we attempted to elucidate the mechanisms of the oviductal development.

The oviduct consists of a monolayer of epithelium and surrounding mesenchyme with muscle layers. The epithelium is occupied by two types of cells: secretory cells and ciliated cells. Their initial appearance indicates that determination to secretory cells or ciliated cells takes place at P3∼5. The developmental progress in the oviduct seems to be orchestrated by events proceeding in the uterus and vagina at the neonatal stage [5–7]. The present study shows that the distribution pattern of the two types of epithelial cells is not changed by the estrus cycle in any region of the adult oviduct (Fig. 2G). This observation is supported by estrogen receptor α-independent ciliogenesis in the oviductal epithelium [4]. Therefore, the results deny the effects of ovarian hormones on the ratio and distribution pattern of epithelial cell populations.

Recombinants of epithelial and mesenchymal tissues developed miniature oviducts when grafted under the kidney capsule. The enzymatic treatment resulted in clean separation of epithelium and mesenchyme as confirmed histologically. In addition, no oviductal structures are reconstructed when any piece of epithelial or mesenchymal tissue is singly grafted, ruling out the possibility that unseparated cells contribute to reconstruction of oviductal structures. Approximately 10% of epithelial cells in reconstructed oviducts are negative for both β-tubulin IV and OVGP1. Experimental procedures may cause damage to tissues. For instance, enzymatic treatments will destroy surface structures of epithelial and mesenchymal cell membrane.

In homotypic recombinants (P3-AmpM and P3-AmpE as well as P3-IstM and P3-IstE), epithelia took the region-specific distribution pattern of two types of epithelial cells. In heterotypic recombinants (P3-AmpM and P3-IstE as well as P3-IstM and P3-AmpE), the distribution pattern of two types of epithelial cells became dependent on the regional origin of the mesenchyme. These results indicate that the mesenchyme determines the fate of the undetermined epithelial cells, that is, either to secretory cells or ciliated cells. At P3, the determining mechanism is activated because some epithelial cells in the ampullar region are OVGP1 positive, but the immunoreactivity might not be an indication of the final determination. They are still plastic and can change to the other type of cells when combined with heterotypic mesenchyme. Our previous study demonstrated that mesenchymal cell populations are functionally linked with epithelial cell populations in the mouse oviduct [9], suggesting that the mesenchyme is made up of mixed populations locally and preferentially regulates epithelial proliferation and function. In addition, mesenchymal cells are also in the course of development at the postnatal period. Therefore, the determining mechanism is not a simple process, but rather a series of developmental events.

Cell-cell interaction in organogenesis is called ''secondary (reciprocal) induction,'' a process that has been extensively studied since the 1950s. Various tissue anlages were enzymatically separated into epithelia and mesenchyme. The epithelia alone or in combination with homologous or heterologous mesenchyme were then cultured in vitro or grafted into the anterior eye chamber or under the kidney capsule. These studies concluded that the mesenchyme plays a critical role in the organogenesis of the kidney [10], pancreas

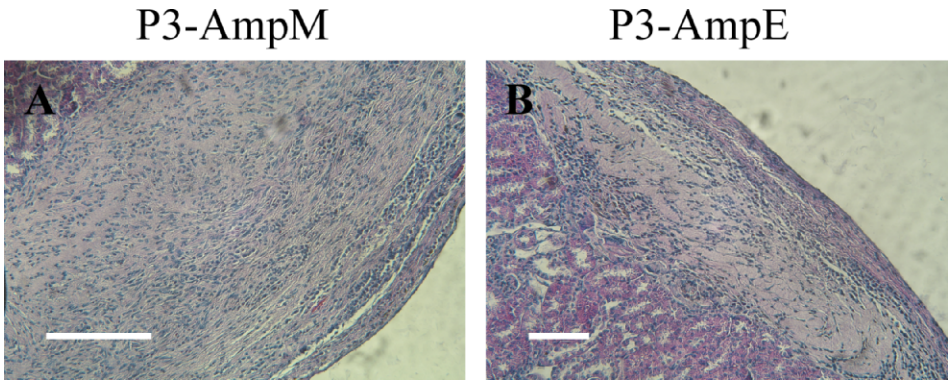

FIG. 5. Examination of possible residual cells with separated tissues. P3-AmpM (**A**) and P3-AmpE (**B**) were singly grafted under kidney capsule for 4 wk. Cell clusters were observed. No oviductal structures were reconstructed. Bar = 100 μm.

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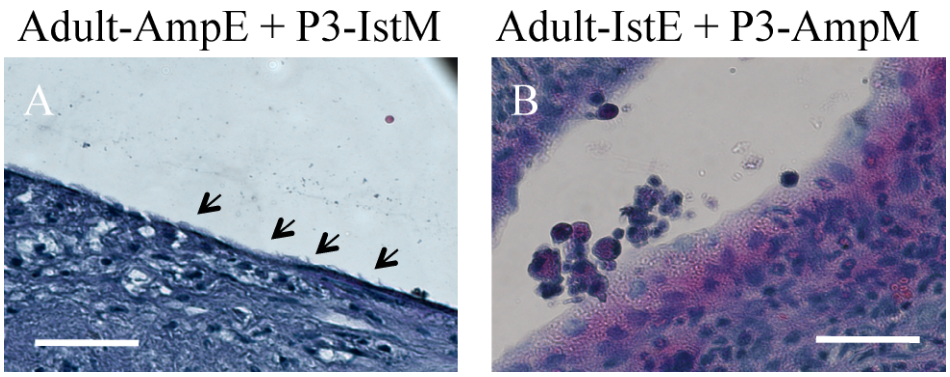

FIG. 6. Fate determination is irreversible. Recombined tissues were implanted under a kidney capsule for 4 wk. **A**) Adult-AmpE recombined with P3-IstM developed epithelia with numerous cilia (arrows). **B**) Adult-IstE recombined with P3-AmpM developed epithelia of simple columnar cells. Bar = 100 μm.

[11, 12], tooth [13, 14], mammary gland [15], lung [16], and gastrointestine [17]. As shown in the present study, the tissue recombinant method has been successfully adapted for the study of the biology of the oviduct, and the results also demonstrate that the mesenchyme plays critical roles in organogenesis of the mouse oviduct.

In the 1970s, the search for mesenchymal factors was begun, and at present, the attempt has been without success [18–20]. One of the major obstacles in the search for mesenchymal factors is the fact that developmental events occur three-dimensionally during a brief period in tissues at small scales. Immortalized cell lines with distinct phenotypes would circumvent the difficulties. We have already demonstrated that the strain of *Trp53*$^{-/-}$ mouse is a useful source for establishing clonal cell lines from various tissues, including the oviduct [21–25], and that the established cell lines express developmental phenotypes [9, 26–28]. Oviductal cell lines allow us to reconstruct models in which we can analyze epithelial-mesenchymal interactions. Therefore, the recombinant method and oviductal cell lines are useful tools to isolate and identify the mesenchymal factors.

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