Role of foxj1 and estrogen receptor alpha in ciliated epithelial cell differentiation of the neonatal oviduct

A Okada^{1,2}, Y Ohta^{2,6}, S L Brody³, H Watanabe^{5,6}, A Krust⁴, P Chambon⁴ and T Iguchi^{5,6}

¹Safety Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd, Tokyo 174-8511, Japan

²Department of Veterinary Science, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

³Department of Internal Medicine, Washington University School of Medicine, St Louis, Missouri 63110-1093, USA

⁴Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, 67404 Illkirch Cedex, France

⁵Center for Integrative Bioscience, Okazaki National Research Institutes, and Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies, Okazaki 444-8585, Japan

⁶CREST, Japan Science and Technology, Kawaguchi 332-0012, Japan

(Requests for offprints should be addressed to T Iguchi, 5-1 Higashiyama, Myodaiji, Okazaki 444-8585, Japan; Email: taisen@nibb.ac.jp)

Abstract

Estrogen regulates proliferation and differentiation of epithelial cells in the mammalian oviduct, but pathways for cell-specific differentiation are not well understood. In the epithelial cells of the developing rat oviduct, we found estrogen receptor (ER) α is expressed at birth and persists in all cells through neonatal day (ND) 7 when ciliated cells appear. To determine a specific function of ER and foxj1, a transcription factor known to have fundamental roles in ciliogenesis in the lung, in differentiation of the ciliated epithelial cells, we treated newborn rats from ND 0 to 5 with estradiol-17 β (E2) with and without a selective ER antagonist. E2 enhanced the number of proliferating cells and accelerated the process of epithelial cell differentiation resulting in ciliogenesis by ND 5, and co-treatment with an ER antagonist inhibited these changes. Foxj1 was expressed only in the infundibulum and ampulla (INF/AMP). That expression preceded the appearance of cilia and was induced by E2. Cilia were absent in oviducts of foxj1-deficient mice, indicating that foxj1 plays a critical role in oviductal ciliogenesis. However, we found the presence of cilia in the ER α -deficient mouse oviduct. The widespread expression of ER α in oviductal epithelium, but restriction of cilia to the INF/AMP regions, and importantly, the presence of cilia in the ER α -deficient mice, suggested ER signaling is not essential for ciliated epithelial cell differentiation. These observations demonstrate that, although E2 stimulates the differentiation process of ciliated epithelial cells, foxj1 is directly required for epithelial cell ciliogenesis of the neonatal oviduct.

Journal of Molecular Endocrinology (2004) **32**, 615–625

Introduction

The mammalian oviduct or fallopian tube has fundamental roles in gamete transport, fertilization, and early embryo development that are facilitated by ciliated and secretory epithelial cells lining the lumen. Several experimental models have shown that oviductal development is regulated by the ovarian sex steroid hormones estrogen and progesterone (Jansen 1984). In the chick and mouse oviduct, estradiol-17 β (E2) induces epithelial cell proliferation and differentiation (Anderson & Hein 1976). In this regard, the differentiation of the

secretory epithelial cell phenotype to the ciliated epithelial cell phenotype can be induced by E2 in primary cultured human fallopian tube epithelial cells (Comer *et al.* 1998). In addition, the appearance of oviductal epithelial cilia, typically observed at day 5 after birth (Komatsu & Fujita 1978), can be accelerated by treatment with E2 in neonatal mouse and hamster oviducts (Eroschenko 1982, Abe & Oikawa 1993). Thus E2 must activate critical pathways required for ciliated epithelial cell differentiation in the oviduct.

E2 action is mediated by its cognate receptors, estrogen receptor (ER) α and ER β (Mangelsdorf

et al. 1995). In developing mice and rats, ER α is expressed in oviductal epithelial and stromal cells, but ER β is absent in both cell types (Yamashita *et al.* 1989, Li 1994, Mowa & Iwanaga 2000, Okada *et al.* 2002a, 2003). Pre- and neo-natal exposure to diethylstilbestrol (DES), a synthetic estrogen, caused abnormal morphology and altered cell proliferation in mouse and rat oviducts (Newbold *et al.* 1983, Okada *et al.* 2001). However, the oviduct of mice lacking ER α was resistant to neonatal DES exposure (Couse *et al.* 2001) indicating an essential role of ER α in mediating the estrogen effect on developing neonatal oviduct. Together, these findings suggest that differentiation and proliferation of epithelial cells in the oviduct may be regulated by E2 through ER α . However, the molecular and cellular mechanisms of these events and factors determining the differentiation of ciliated and non-ciliated cells of the oviduct have not been clarified.

Foxj1 (formerly HFH-4) is a member of the fork head box (fox) family of transcription factors with fundamental roles in embryonic development and differentiation (Carlsson & Mahlapuu 2002). Foxj1 has been cloned from mice, rats and humans, and is shown to have expression restricted to ciliated epithelial cells of conducting airways, choroid plexus, and ependyma of the brain, the testis, and the oviduct (Clevidence *et al.* 1993, Hackett *et al.* 1995, Brody *et al.* 1997, Lim *et al.* 1997, Murphy *et al.* 1997, Blatt *et al.* 1999). In the developing mouse lung, the onset of foxj1 expression has been observed from embryonic day 14.5 to 16.5, prior to the appearance of cilia on epithelial cells (Hackett *et al.* 1995, Blatt *et al.* 1999, Tichelaar *et al.* 1999a). Foxj1 gene interruption in mice (foxj1KO) results in perinatal lethality and a lack of ciliated epithelial cells in tissues including the lung and oviduct (Chen *et al.* 1998, Brody *et al.* 2000). Transgenic mice overexpressing foxj1 under control of the surfactant protein C promoter in the lung show induction of ciliogenesis in undifferentiated alveolar epithelial cells (Tichelaar *et al.* 1999b) as demonstrated by the expression of the foxj1 transgene and β -tubulin IV, a ciliated epithelial cell marker (Renthal *et al.* 1993). These findings indicate that foxj1 has a distinct role in ciliated epithelial cell differentiation in the embryonic through the adult lung. However, the role of foxj1 in the development and differentiation of oviductal ciliated epithelial cells, which are regulated by sex steroid hormones, has not been

established. The restricted expression of foxj1 in ciliated cells of the oviduct and a lack of oviductal cilia exhibited in foxj1KO mice led us to hypothesize that foxj1 has a critical role in the E2-dependent ciliated epithelial cell differentiation of the oviduct.

This study shows for the first time that E2 exposure results in the induction of foxj1 in ciliated cells during ER signaling in the neonatal rat oviduct. Interestingly, ER α is down-regulated in the ciliated cells following E2 exposure and the epithelial cells in the mouse oviduct of the null mutant for ER α (ER α KO) have normal foxj1 expression and cilia, suggesting that ER signaling is not directly required for oviductal epithelial cell ciliogenesis. However, evaluation of the foxj1KO mouse oviduct indicates that foxj1 is required for cell-specific down-regulation of ER α and ciliogenesis in ciliated epithelial cells of the neonatal oviduct. Therefore, foxj1 has a critical role in directing oviductal epithelial cell differentiation.

Materials and methods

Animals and treatments

All animals were maintained in accordance with the NIH and the Institutional Guides for the Care and Use of Laboratory Animals. Male and female Sprague–Dawley rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Animals were housed individually in stainless-steel cages with controlled temperature ($23 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$), and a 13 h light:11 h darkness cycle (0800–2100 h). Pelleted food (CRF-1; Oriental Yeast Co., Ltd, Tokyo, Japan) and municipal tap water were freely available. Females (15 weeks of age) were cohabited overnight with males, and the day on which neonates were born was designated as neonatal day (ND) 0. In ontogenic rat studies, oviducts were removed from untreated neonatal rats on ND 0, 3, 5, 7, 10 and 20. Foxj1KO (Brody *et al.* 2000) and ER α KO (Dupont *et al.* 2000) mice, and their wild-type (WT) counterparts (C57/BL/6) were used at 3 weeks and 2 months of age respectively.

To investigate the effect of E2, 16–28 female neonates in each group were injected s.c. daily with sesame oil, or 1 or 10 μg of E2 (Sigma, St Louis, MO, USA) for 5 days (ND 0–4). Similarly, sesame oil or 10 μg of ICI 182780 (ICI) (Tocris Cookson

Inc., Ballwin, MO, USA) was concomitantly injected for 5 days (ND 0–4). Oviducts were removed 24 h after the last injection (ND 5), and immediately prepared for immunohistochemistry or real-time RT-PCR. All chemicals were dissolved in sesame oil and injected at a dosing volume of 0.05 ml per neonate.

Total RNA preparation and real-time RT-PCR

Procedures for total RNA preparation and real-time RT-PCR have been described previously (Okada *et al.* 2002b). Total RNA was isolated from oviducts, and reverse-transcribed into cDNA. An aliquot of generated cDNA was amplified with a pair of primers (foxj1, forward 5'CCAAGAATTG GCAAAGCA3' and reverse 5'TTTCTCTGGG TAGGGACCTG3'; and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), forward 5'TCTACCC ACGGCAAGTTCAAT3' and reverse 5'ACCCC ATTTGATGTTAGCGG3') derived from rat mRNA sequences (GenBank Accession No. L36388 and M17701 respectively). Quantitative real-time PCR was carried out in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix reagent (Applied Biosystems). PCR cycle parameters were 94 °C for 15 s, 60 °C for 30 s and 72 °C for 60 s, following a hold temperature for 10 min at 95 °C. The number of template copies present at the start of the reaction was determined by comparison with a standard scale prepared from rat genomic DNA. The expression level of each target gene was calculated by standardizing the target gene copy number with the GAPDH copy number in a sample. Purity and specificity of the PCR products were confirmed by the omission of the reverse transcriptase, having a single melting temperature, being of the appropriate size and having the proper sequence. The results of analysis is based on duplicate samples from three independent experiments.

Antibodies

A rabbit polyclonal antibody against foxj1 was generated as described previously (Blatt *et al.* 1999). A mouse monoclonal antibody against ER α (6F11; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) was used at a dilution of 1:50. Mouse monoclonal antibodies against proliferating cell

nuclear antigen (PCNA) (PC10; Dako Corporation, Carpinteria, CA, USA) and β -tubulin IV (ONS1A6; BioGenex, San Ramon, CA, USA) were used at a dilution of 1:100 and 1:250 respectively. The binding specificity of these antibodies has been previously established (Bannerjee *et al.* 1992, Fisher *et al.* 1997, Blatt *et al.* 1999, Pelletier *et al.* 2000).

Tissue preparation and immunohistochemistry

Oviducts were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C. Sections cut in paraffin at 4 μ m were deparaffinized, rehydrated and autoclaved at 121 °C for 15 min in 10 mM citrate buffer, at pH 6.0, for antigen retrieval. Sections were then rinsed in distilled water and incubated with 0.3% hydrogen peroxide for 30 min. After rinsing in 0.01% Triton X-100 in PBS (PBT), sections were treated with normal sheep serum (Dako) for 30 min, and then incubated for 30 min with anti-PCNA antibody, or for 16 h at 4 °C with anti-foxj1, -ER α , or - β -tubulin IV antibodies. Sections were rinsed in PBT and treated with Simple Stain Rat PO (Nichirei, Tokyo, Japan) for 30 min. After a final PBT wash, sections were treated with 0.01% 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl at pH 7.6 including 0.068% imidazole (Sigma) and 0.02% hydrogen peroxide for 5 min.

For double immunohistochemistry, sections stained for ER α or PCNA as described above were rinsed in PBT and blocked in normal sheep serum, followed by incubation with the anti- β -tubulin IV antibody overnight at 4 °C. Sections were rinsed in PBT and treated with EnVision/AP (Dako) for 30 min. After rinsing in PBT, they were treated with fuchsin (Dako) with levamisole (Dako) for 5 min.

All sections, except those for the ontogeny study, were lightly counter-stained with hematoxylin (Dako A/S, Glostrup, Denmark). Normal mouse IgG and normal rabbit immunoglobulin fraction (both Dako A/S) were used as negative controls in place of primary antibodies for ER α , PCNA, β -tubulin IV and foxj1 immunostaining respectively, showing no specific immunoreactivity.

Evaluation and statistical analysis

Sections were examined and photographed using a light microscope attached to a digital camera

(BX60 and DP50; Olympus Optical Co., Ltd, Tokyo, Japan). Cell proliferation was calculated as a percentage of the total PCNA-labeled cells counted; all epithelial cells in each section evaluated were counted. For stroma, all cells in the five layers most adjacent to the epithelium in each section were counted. Ciliated cell differentiation was evaluated as a percentage of the total ducts including β -tubulin IV-labeled cells in a section. At least seven specimens from each of five animals were examined for all investigations. Although, following treatment with E2, regional differentiation occurred in the oviduct, PCNA and β -tubulin IV expressions were evaluated throughout oviductal regions.

Statistical analysis was carried out, using Duncan's multiple comparison test, for the effects of hormones on β -tubulin IV, *foxj1* and PCNA expressions. Data are represented as means $\pm$ s.d. and considered significantly different at $P < 0.05$.

Results

Differentiation of ciliated epithelial cells in the untreated-neonatal rat oviduct

To evaluate the differentiation of oviductal epithelial cells, the expression of cilia protein β -tubulin IV was used as a marker of ciliated cells and evaluated by immunohistochemistry during postnatal oviductal development (Fig. 1). The neonatal oviduct is a simple tube structure from ND 0 to ND 5, and subsequently differentiates morphologically into the infundibulum (INF), ampulla (AMP), isthmus (IST) and uterotubal junction (UTJ) after ND 7. No expression of β -tubulin IV was found in the oviduct from ND 0 to 5. However, on ND 7, β -tubulin IV appeared on the luminal surface of some epithelial cells. Following morphological differentiation of the oviduct into different regions, the number of cells expressing β -tubulin IV increased. Ciliated cells were numerous in the INF/AMP on ND 20 and at diestrus of the cycling oviduct. Only rare epithelial β -tubulin IV-positive cells were detected in the IST/UTJ. Thus, consistent with a previous report that used electron microscopy to detect ciliated cells, the appearance of cilia in the oviduct was after ND 5, following morphological development of the oviduct (Komatsu & Fujita 1978), and was restricted to specific regions.

Figure 1 Differentiation of ciliated epithelial cells in the neonatal rat oviduct. Neonatal rat oviduct was evaluated for cilia protein β -tubulin IV using immunohistochemistry. β -Tubulin IV was not detected on ND 5, but was present on ND 7. On ND 20, β -tubulin IV expression was present in epithelial cells of the INF/AMP, but not of the IST/UTJ. In the adult rat oviduct, β -tubulin IV was detected in epithelial cells of the AMP, but not of the IST. Arrowheads indicate β -tubulin IV-positive epithelial cells. Bar: 50 μ m for ND 5, 7 and 20, 100 μ m for adults.

Ontogeny of *foxj1* in the untreated neonatal rat oviduct

Transcription factor *foxj1* is expressed in ciliated cells prior to the appearance of cilia in the airway (Blatt *et al.* 1999). To evaluate changes in expression of *foxj1* mRNA in the developing rat oviduct, quantitative real-time RT-PCR was performed (Fig. 2A). A low level of *foxj1* mRNA was detected in the neonatal oviduct on ND 0 and 3. Oviductal *foxj1* mRNA increased dramatically from ND 5 and reached a high level similar to that in the diestrus oviduct of cycling rats by ND 20.

At the protein level, no expression of *foxj1* was found in oviductal tissue from ND 0 to 5 (Fig. 2B). The onset of *foxj1* expression was on ND 7, at which time protein was detected in the nuclei of epithelial cells. The expression of *foxj1* increased on ND 10 (not shown) and ND 20 in the INF/AMP, and was most striking in the INF/AMP

Figure 2 Foxj1 expression in the oviduct of neonatal rats during differentiation. (A) Oviducts were harvested at indicated NDs and evaluated by real-time RT-PCR for foxj1 mRNA expression standardized to GAPDH mRNA. Foxj1 expression dramatically increased from ND 5 to ND 20 when the level was similar to that in the diestrous oviduct. Data represent the means $\pm$ s.d. of duplicate samples ($n=6-12$) from three independent experiments. (B) Neonatal oviducts were evaluated for expression of foxj1 by immunohistochemistry. Epithelial cell expression of foxj1 was not detected on ND 5, but was present on ND 7. On ND 20, foxj1 was expressed in epithelial cells of the INF/AMP, but not of the IST/UTJ. In the adult rat oviduct, foxj1 was expressed in epithelial cells of the AMP, but not the IST. Arrows indicate foxj1-positive epithelial cells. Bar: 50 μ m for ND 5, 7 and 20, 100 μ m for adults.

at the time of diestrus in the cycling oviduct. No foxj1 expression was detected in the IST/UTJ after morphological differentiation on ND 20 and adult diestrus. The protein level of foxj1 may be low and below the limit of immunohistochemical detection from ND 0 to 5, since its mRNA expression was evident at a low level. Thus, as in the airway of the lung, the expression of foxj1 preceded the appearance of cilia, suggesting that foxj1 similarly regulated ciliogenesis in the oviduct.

Neonatal effects of E2 on ciliated epithelial cell differentiation

To investigate the effects of E2 on neonatal development of the oviduct, oil vehicle or 1 or 10 μ g E2 were injected into neonatal rats for 5 days from the day of birth. Also, oil vehicle or pure ER antagonist ICI was concomitantly injected with 10 μ g E2. Following E2 treatment, changes in the morphology of the primitive oviductal tube into the INF/AMP and IST/UTJ regions were observed on ND 5. Apparent estrogenic effects such as hypertrophy of epithelial and stromal cells, including enlargement of nuclei and dilatation of the lumen in the IST/UTJ were histologically observed (Newbold *et al.* 1983). In the oil-treated oviduct, no duct with cells immunopositive for β -tubulin IV was detected on ND 5, but 68% of ducts had differentiated ciliated epithelial cells on ND 7 (Fig. 3). Following treatment with 1 μ g and 10 μ g E2, 20 and 78% of the ND 5 ducts showed epithelial β -tubulin IV expression respectively. Treatment with ICI significantly reduced the percentage of β -tubulin IV-expressing epithelial cells within the duct to 20% ($P<0.01$) (Fig. 3). From this, it can be deduced that ovarian hormones regulate oviductal morphology and oviductal differentiation, notably affecting differentiation of cells to the ciliated cell phenotype.

Neonatal effects of E2 on foxj1 expression

Next it was determined if the effect of E2 on ciliogenesis was regulated by foxj1. Expression of foxj1 was evaluated in the oil- and hormone-treated oviducts by real-time RT-PCR and immunohistochemistry. Compared with the oil control, E2 treatments of 1 and 10 μ g showed 1.2- and 2.2-fold increases respectively ($P<0.05$) in foxj1 mRNA in the ND 5 oviduct (Fig. 4). Co-treatment with ICI

Figure 3 Effects of E2 on ciliated epithelial cell differentiation in the neonatal oviduct. Neonatal rats were treated with oil control, E2, or E2 plus ICI and evaluated by immunohistochemistry for β -tubulin IV expression in ND 5 oviduct. E2 (1 and 10 μ g) significantly induced β -tubulin IV expression in ND 5 oviduct. A concomitant treatment with ICI significantly inhibited 10 μ g E2-induced β -tubulin IV expression. Data shown are the means $\pm$ s.d. of cells expressing β -tubulin IV in at least seven specimens from five animals in three independent experiments. * P <0.01 vs Oil+E2-1, # P <0.01 vs Oil+E2-10. a, Statistical analysis not conducted.

Figure 4 Effects of E2 on foxj1 expression in neonatal oviduct. Neonatal rats were treated with oil control, E2, or E2 plus ICI as in Fig. 3, and ND 5 oviduct mRNA was evaluated for foxj1 expression by real-time RT-PCR. The expression level in the oil-treated group was designated as 100% and compared with the relative expression levels observed in other samples. E2 (1 and 10 μ g) significantly induced foxj1 expression in the ND 5 oviduct. A concomitant treatment with ICI significantly inhibited 10 μ g E2-induced foxj1 expression. Data represent the means $\pm$ s.d. from three independent experiments composed of 14–21 neonates each. * P <0.05 vs Oil, \$ P <0.05 vs Oil+E2-1, # P <0.05 vs Oil+E2-10. a, Statistical analysis not conducted.

reduced 10 μ g E2-induced foxj1 expression to the oil-treated control level. Although, on ND 5, no foxj1-positive cell was detected in the oil-treated oviduct, E2 induced foxj1 expression in epithelial cells in the INF/AMP, but not in the IST/UTJ region (data not shown). Some of the foxj1-positive cells in the E2-treated INF/AMP had cilia, but others did not. These data suggested that foxj1 expression was regulated by E2.

Neonatal effects of E2 on cell proliferation

E2 treatment is known to be associated with oviductal epithelial cell proliferation as well as differentiation. To determine if E2 directed differentiation of oviductal epithelial cells by altering cell phenotype directly or alternatively by inducing proliferation that was followed by differentiation, the relationship between markers of proliferation and ciliogenesis in the oviductal epithelium was examined. The effect of E2 on cell proliferation was determined by immunohistochemical detection of PCNA (Fig. 5). On ND 5, the control oil-treated oviduct had 16% PCNA-positive

epithelial cells, but the 1 and 10 μ g E2 treatment significantly increased proliferation to 22% (P <0.05) and 35% (P <0.01) respectively. In the E2-treated tissues, epithelial PCNA was observed in most epithelial cells of the IST/UTJ, but in only some cells of the INF/AMP. Interestingly, PCNA-positive epithelial cells in the INF/AMP region lacked β -tubulin IV expression. Conversely, β -tubulin IV-expressing epithelial cells did not co-express PCNA in the E2-treated oviduct. Concomitant treatment with ICI caused a decrease in the percentage of 10 μ g E2-enhanced PCNA-labeled epithelial cells to the control level. These findings might suggest that, in the INF/AMP, E2 induced proliferation of oviductal epithelial cells that subsequently differentiate into ciliated cells.

Neonatal effects of E2 on ER expression

Variation in regional oviductal proliferation and differentiation following E2 treatment suggested that the expression of sex steroid hormone receptors was also cell- and region-specific. We

therefore investigated the effects of E2 on the expression of ER α and ER β in oil- and hormone-treated oviducts. In the control oil-treated ND 5 oviduct, there was marked expression of ER α in the nuclei of epithelial and stromal cells (Fig. 6). Following exposure to 1 and 10 μ g of E2, the expression of epithelial ER α in both the INF/AMP

and IST/UTJ was less detectable at ND 5. Absent receptor expression in ciliated epithelial cells expressing β -tubulin IV of the INF/AMP was noted. Similar epithelial cell expression patterns of ER α were detected in the adult rat oviduct, including the expression in all epithelial cells of the IST, but not in ciliated epithelial cells of the AMP (Fig. 6) as reported (Okada *et al.* 2003). However, stromal ER α was not changed by E2, and maintained a control level by co-exposure to ICI (not shown). Expression of ER β was not detected in the oil-treated oviduct as previously reported (Okada *et al.* 2003), or in the E2-treated oviduct evaluated on ND 5 (data not shown). Taken together, these findings indicate a dynamic change in ER α expression during oviductal growth and differentiation that is cell- and region-specific. The initial presence of ER α in all AMP epithelial cells, followed by loss of expression in ciliated cells, might suggest that E2 was required for a proliferative phase that precedes a ciliogenesis phase.

Oviductal epithelial cell differentiation in the foxj1- and ER α -deficient mice

To understand the role of foxj1 and ER α in the development and differentiation of the mammalian oviduct, the expression of ciliogenesis markers and ER α were evaluated in oviducts from genetically

Figure 5 Effects of E2 on epithelial cell proliferation in neonatal oviduct. Neonatal rats were treated with oil control, E2, or E2 plus ICI as in Fig. 3 and epithelial cell proliferation in the ND 5 oviduct was evaluated by immunostaining for PCNA expression. (A) There was a significant increase in the number of cells expressing PCNA in the oviducts of the E2 (1 and 10 μ g)-treated mice compared with controls. A concomitant treatment with ICI significantly inhibited 10 μ g E2-stimulated PCNA expression. Data represent the means $\pm$ S.D. of epithelial cells expressing PCNA from 14–21 neonates in three independent experiments. ** P < 0.01 and * P < 0.05 vs Oil, \$ P < 0.01 vs Oil+E2-1, # P < 0.01 vs Oil+E2-10. (B) Double immunostaining for expression of PCNA (brown) and β -tubulin IV (red) in the oil-treated oviduct (Oil), and the 10 μ g E2-treated INF/AMP and IST/UTJ. Ten micrograms of E2 induced both PCNA and β -tubulin IV expression in some epithelial cells of the INF/AMP, but only PCNA in most epithelial cells of the IST/UTJ. In E2-treated INF/AMP, PCNA expression was not observed in cells that expressed β -tubulin IV (arrowheads). Expression was not detected in samples immunostained using normal mouse IgG (Control). Bar: 50 μ m.

Figure 6 Effect of E2 on ER α expression during neonatal oviductal epithelial cell differentiation. Neonatal rats were treated with oil control, or E2 as in Fig. 3 and ER α (brown) and β -tubulin IV (red) expression evaluated by double immunohistochemistry on ND 5. Representative images are shown in the oil-treated oviduct (Oil), 10 μ g E2-treated INF/AMP and IST/UTJ, untreated adult AMP and IST, and immunostaining with normal mouse IgG (Control). E2 reduced ER α expression in epithelial cells of both the INF/AMP and IST/UTJ. Importantly, after E2 treatment, ER α was absent in ciliated epithelial cells expressing β -tubulin IV of the INF/AMP. The adult rat oviduct displayed similar expression patterns of epithelial ER α , showing the expression in all epithelial cells of the IST, but not in ciliated epithelial cells of the AMP. Expression was not detected in control samples (Control). Bar: 50 μ m.

deficient foxj1 (foxj1KO) and ER α (ER α KO) mice. In the WT mouse oviduct, β -tubulin IV and foxj1 were expressed in ciliated epithelial cells, as in the untreated rat oviduct (Fig. 7). In the foxj1KO oviduct, no foxj1 or β -tubulin IV was observed as had been previously reported (Chen *et al.* 1998, Brody *et al.* 2000). Remarkably, the absence of foxj1 also resulted in persistent expression of ER α in all cells of the AMP, compared with down-regulation of this protein seen in the ciliated cells of WT oviducts. Furthermore, ciliogenesis did not require an intact ER α because in ER α KO mice, epithelial foxj1 and β -tubulin IV were detected in

the INF/AMP as in WT mice (Fig. 7). Thus foxj1 is required for ciliogenesis and continued maturation of the oviductal epithelium supporting a critical role for foxj1 in oviductal differentiation.

Discussion

In this report, it has been shown that oviductal epithelial cell differentiation, and particularly ciliogenesis in the developing oviduct, is dependent on fork head transcription factor foxj1. We found that, during normal rat oviductal epithelial cell differentiation, foxj1 mRNA was observed at low levels, even on ND 0, then increased significantly between days 5 and 7. At ND 7 foxj1 protein was first detected immunohistochemically in epithelial cells of the INF/AMP. Coincident with foxj1 expression was the appearance of cilia protein β -tubulin IV, suggesting that epithelial differentiation into ciliated epithelial cells occurred between ND 5 and ND 7. These findings are in agreement with a previous investigation of mouse oviductal epithelial cell differentiation that utilized electron microscopy to demonstrate the onset of ciliogenesis on ND 5 and ongoing differentiation from days 5 to 10 (Komatsu & Fujita 1978). The expression level of foxj1 protein may be so low, from ND 0 to 5, to be below the limit of immunohistochemical detection. Therefore, in the developing oviduct, as well as the developing lung (Hackett *et al.* 1995, Tichelaar *et al.* 1999a), foxj1 may be expressed before the appearance of epithelial cilia formation. Thus, consistent with previous reports of foxj1 function and the absence of cilia in the oviduct in foxj1 null mice, our findings indicate a key role for foxj1 in the ciliogenesis of the oviduct. Also considered were previous reports that indicated that estrogen signaling may be critical for ciliogenesis, thereby suggesting that E2 could activate foxj1 (Anderson & Hein 1976, Eroschenko 1982, Jansen 1984, Abe & Oikawa 1993, Comer *et al.* 1998).

It was found that E2 treatment of neonatal rat oviducts caused changes in oviductal morphology and the expression of differentiation markers, inducing ciliated cells at ND 5. In the INF/AMP region, five daily injections of E2 induced foxj1 and β -tubulin IV. To determine the function of E2 in specific cell types during differentiation, the expression of ER α during normal oviductal

Figure 7 Effect of *foxj1* on oviductal epithelial cell differentiation. Expression of β -tubulin IV, *foxj1* and *ERα* was evaluated by immunohistochemistry in WT, *foxj1* KO (*foxj1*^{-/-}), and *ERα* KO (*ERα*^{-/-}) oviduct. β -tubulin IV and *foxj1* were expressed in similar patterns in WT and *ERα*^{-/-} oviduct. Epithelial cells in the AMP of the WT oviduct showed marked *ERα* expression in non-ciliated cells but little expression in ciliated cells. In the *foxj1*^{-/-} oviduct, *ERα* protein was highly expressed in all epithelial cells (all non-ciliated cells). Arrowheads indicate ciliated epithelial cells. Bar: 20 μ m.

differentiation and E2 treatment was examined. At ND 5, expression of *ERα* was markedly expressed in both epithelial and stromal cells of the oil-treated oviduct. A pure ER antagonist, ICI, inhibited all E2 effects observed, indicating the dependence of these E2 actions on ER signaling. However, all changes in gene expression associated with E2 treatment were similar to changes observed in the untreated oviduct at ND 7 or later, and several additional observations suggests that E2 is not required for ciliogenesis. First, although E2 affects proliferation throughout the oviduct, ciliogenesis occurs regionally, i.e. only within the INF/AMP region and not in the IST/UTJ, suggesting that *foxj1* was regulated through a different pathway. Secondly, cells expressing *foxj1* lost *ERα* and progesterone receptor (A Okada, Y Ohta and T Iguchi unpublished observations) expression. This could be an event that follows ciliogenesis after induction by E2. Thirdly, epithelial differentiation including *foxj1* expression and ciliogenesis was normal, however, in the *ERα* KO oviduct. Therefore, ER signaling is not essential for ciliated epithelial differentiation during the neonatal development of the oviduct. Also consistent with this is the absence of estrogen-responsive elements in the promoter region of the *foxj1* gene. Thus, although

E2 accelerated differentiation of ciliated epithelial cells, any molecular mechanisms for regulation of *foxj1* by E2 remain to be clarified.

While *ERα* signaling in the ciliated cell may not be required for the direct induction or maintenance of the ciliated cell phenotype, it is reasonable to assume that E2-mediated *ERα* signaling functions to activate progenitor cell proliferation and to subsequently generate cells with the capacity to achieve the ciliated cell phenotype. In this regard, E2 treatment was associated with epithelial cell proliferation in a substantial number of epithelial cells of the INF/AMP and all epithelial cells of the IST/UTJ. In addition, we found ciliated cells were not proliferating and did not express PCNA, behavior consistent with a terminally differentiated cell. Thus, while normal cilia formation in the *ERα* KO oviduct indicates that E2 is not an absolute requirement for epithelial cell differentiation, it may likely be induced via multiple compensatory pathways in the *ERα* KO or WT mouse.

Differentiation of oviductal epithelial cells is induced by E2, but in later stages it is independent of E2 and is instead dependent on *foxj1*. In the *foxj1* KO mouse, a regional difference between the INF/AMP and the IST/UTJ was easily recognized

light-microscopically, and ER α and progesterone receptor (A Okada, Y Ohta, S L Brody and T Iguchi unpublished observations) were present in all epithelial cells in the INF/AMP region. These findings may indicate that epithelial cells in the foxj1KO oviduct have ceased their differentiation into ciliated epithelial cells due to a lack of foxj1. It is also likely that foxj1 has a much broader role than only the regulation of ciliogenesis and instead is required to switch off the E2 (and other) pathways. In addition to foxj1, other fox genes for sex steroid hormone actions have regulatory roles in reproductive tissues. Foxo1 (FKHR, forkhead homologue of rhabdomyosarcoma), foxo3 (FKHRL1, forkhead-like protein-1), and foxo4 (AFX, a forkhead transcription factor) are expressed in the rodent ovary at specific stages and in specific cells of follicular development and luteinization (Schoor *et al.* 2001, Zhao *et al.* 2001), and foxo1 is regulated by E2 in the granulosa cells (Richards *et al.* 2002). Additionally, foxa1 (HNF-3 α) is expressed in the epithelia of the rat prostate and, 7 days following castration, was suppressed in the prostate but restored by testosterone (Kopachik *et al.* 1998), indicating an important role in development and maintenance of male urogenital tract epithelial cells. Taken together, forkhead transcription factors including foxj1 may regulate sex steroid hormone actions in development, cytodifferentiation and physiological function of reproductive tissues in both males and females.

In summary, this study proposes important mechanisms in the development of the neonatal rat oviduct. Neonatal effects of E2 on epithelial cell differentiation or proliferation were demonstrated to vary inversely in different oviductal regions and cell types. We hypothesize that in early neonatal development, initiation of E2 production from the ovary as well as an increase in oviductal ER α expression occur, and E2 induces foxj1 expression in programmed ciliated cells of the INF/AMP with a loss of ER α that sequentially promotes epithelial ciliogenesis. However, although the stimulation of ER signaling accelerates the differentiation process of ciliated epithelial cells, the presence of cilia in the ER α KO oviduct suggests that it is not fundamentally required for this event. This study suggests that signaling of foxj1 is directly required for oviductal epithelial cell ciliogenesis, and serves as fundamental evidence for understanding molecular and cellular mechanisms of epithelial cell

differentiation and proliferation of the neonatal rat oviduct.

Acknowledgements

The authors gratefully acknowledge Ms Atsuko Suzuki and Ms Mayu Goto for maintaining and genotyping the ER α KO mice, and Emeritus Professor Noboru Takasugi for critical reading of the manuscript. The authors also thank members of the Molecular Medicine Laboratories and the Safety Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd for their helpful support and comments. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (A) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; an Environmental Research Grant from the Ministry of the Environment; and a Health Sciences Research Grant from the Ministry of Health, Labor and Welfare, Japan.

References

- Abe H & Oikawa T 1993 Effects of estradiol and progesterone on the cytodifferentiation of epithelial cells in the oviduct of the newborn golden hamster. *Anatomical Record* **235** 390–398.
- Anderson RGW & Hein CE 1976 Estrogen dependent ciliogenesis in the chick oviduct. *Cell and Tissue Research* **171** 459–466.
- Bannerjee A, Roach MC, Trcka P & Luduena RF 1992 Preparation of a monoclonal antibody specific for the class IV isotype of β -tubulin. Purification and assembly of $\alpha\beta_{II}$, $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ tubulin dimers from bovine brain. *Journal of Biological Chemistry* **267** 5625–5630.
- Blatt EN, Yan XH, Wuerffel MK, Hamilos DL & Brody SL 1999 Forkhead transcription factor HFH-4 expression is temporally related to ciliogenesis. *American Journal of Respiratory Cell and Molecular Biology* **21** 168–176.
- Brody SL, Hackett BP & White RA 1997 Structural characterization of the mouse *Hfh4* gene, a developmentally regulated forkhead family member. *Genomics* **45** 509–518.
- Brody SL, Yan XH, Wuerffel MK, Song SK & Shapiro SD 2000 Ciliogenesis and left-right axis defects in forkhead factor HFH-4-null mice. *American Journal of Respiratory Cell and Molecular Biology* **23** 45–51.
- Carlsson P & Mahlapuu M 2002 Forkhead transcription factors: key players in development and metabolism. *Developmental Biology* **250** 1–23.
- Chen J, Knowles HJ, Hebert JL & Hackett BP 1998 Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left-right asymmetry. *Journal of Clinical Investigation* **102** 1077–1082.
- Cleveland DE, Overdier DG, Tao W, Qian X, Pani L, Lai E & Costa RH 1993 Identification of nine tissue-specific transcription factors of the hepatocyte nuclear factor 3/forkhead DNA-binding domain family. *PNAS* **90** 3948–3952.
- Comer MT, Leese HJ & Southgate J 1998 Induction of a differentiated ciliated cell phenotype in primary cultures of Fallopian tube epithelium. *Human Reproduction* **13** 3114–3120.

- Couse JF, Dixon D, Yates M, Moore AB, Ma L, Maas R & Korach KS 2001 Estrogen receptor- α knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract. *Developmental Biology* **238** 224–238.
- Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors α (ER α) and β (ER β) on mouse reproductive phenotypes. *Development* **127** 4277–4291.
- Eroschenko VP 1982 Surface changes in oviduct, uterus and vaginal cells of neonatal mice after estradiol-17 β and the insecticide chlordane (Kepone) treatment: a scanning electron microscopic study. *Biology of Reproduction* **26** 707–720.
- Fisher JS, Millar MR, Majdic G, Saunders PTK, Fraser HM & Sharpe RM 1997 Immunolocalisation of oestrogen receptor- α within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *Journal of Endocrinology* **153** 485–495.
- Hackett BP, Brody SL, Liang M, Zeitz ID, Bruns LA & Gitlin JD 1995 Primary structure of hepatocyte nuclear factor/forkhead homologue 4 and characterization of gene expression in the developing respiratory and reproductive epithelium. *PNAS* **92** 4249–4253.
- Jansen RPS 1984 Endocrine response in the fallopian tube. *Endocrine Reviews* **5** 525–551.
- Komatsu M & Fujita H 1978 Electron-microscopic studies on the development and aging of the oviduct epithelium of mice. *Anatomy and Embryology* **152** 243–259.
- Kopachik W, Hayward SW & Cunha GR 1998 Expression of hepatocyte nuclear factor-3 α in rat prostate, seminal vesicle, and bladder. *Developmental Dynamics* **211** 131–140.
- Li S 1994 Relationship between cellular DNA synthesis, PCNA expression and sex steroid hormone receptor status in the developing mouse ovary, uterus and oviduct. *Histochemistry* **102** 405–413.
- Lim L, Zhou H & Costa RH 1997 The winged helix transcription factor HFH-4 is expressed during choroid plexus epithelial development in the mouse embryo. *PNAS* **94** 3094–3099.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P *et al.* 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839.
- Mowa CN & Iwanaga T 2000 Developmental changes of the oestrogen receptor- α and - β mRNAs in the female reproductive organ of the rat – analysis by *in situ* hybridization. *Journal of Endocrinology* **167** 363–369.
- Murphy DB, Seemann S, Wiese S, Kirschner R, Grzeschik KH & Thies U 1997 The human hepatocyte nuclear factor 3/fork head gene FKHL13: genomic structure and pattern of expression. *Genomics* **40** 462–469.
- Newbold RR, Bullock BC & McLachlan JA 1983 Exposure to diethylstilbestrol during pregnancy permanently alters the ovary and oviduct. *Biology of Reproduction* **28** 735–744.
- Okada A, Sato T, Ohta Y, Buchanan DL & Iguchi T 2001 Effect of diethylstilbestrol on cell proliferation and expression of epidermal growth factor in the developing female rat reproductive tract. *Journal of Endocrinology* **170** 539–554.
- Okada A, Ohta Y, Buchanan DL, Sato T, Inoue S, Hiroi H, Muramatsu M & Iguchi T 2002a Changes in ontogenetic expression of estrogen receptor α and not of estrogen receptor β in the female rat reproductive tract. *Journal of Molecular Endocrinology* **28** 87–97.
- Okada A, Ohta Y, Buchanan DL, Sato T & Iguchi T 2002b Effect of estrogens on ontogenetic expression of progesterone receptor in the fetal female rat reproductive tract. *Molecular and Cellular Endocrinology* **195** 55–64.
- Okada A, Ohta Y, Inoue S, Hiroi H, Muramatsu M & Iguchi T 2003 Expression of estrogen, progesterone and androgen receptors in the oviduct of developing, cycling and pre-implantation rats. *Journal of Molecular Endocrinology* **30** 301–315.
- Pelletier G, Labrie C & Labrie F 2000 Localization of oestrogen receptor α , oestrogen receptor β and androgen receptor in the rat reproductive organs. *Journal of Endocrinology* **165** 359–370.
- Renthal R, Schneider BG, Miller MM & Ludueña RF 1993 β_{IV} is the major β -tubulin isotype in bovine cilia. *Cell Motility and the Cytoskeleton* **25** 19–29.
- Richards JS, Sharma SC, Falender AE & Lo YH 2002 Expression of FKHR, FKHL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. *Molecular Endocrinology* **16** 580–599.
- Schuur ER, Loktev AV, Sharma M, Sun Z, Roth RA & Weigel RJ 2001 Ligand-dependent interaction of estrogen receptor- α with members of the forkhead transcription factor family. *Journal of Biological Chemistry* **36** 33554–33560.
- Tichelaar JW, Wert SE, Costa RH, Kimura S & Whitsett JA 1999a HNF-3/forkhead homologue-4 (HFH-4) is expressed in ciliated epithelial cells in the developing mouse lung. *Journal of Histochemistry and Cytochemistry* **47** 823–831.
- Tichelaar JW, Lim L, Costa RH & Whitsett JA 1999b HNF-3/forkhead homologue-4 influences lung morphogenesis and respiratory epithelial cell differentiation *in vivo*. *Developmental Biology* **213** 405–417.
- Yamashita S, Newbold RR, McLachlan JA & Korach KS 1989 Developmental pattern of estrogen receptor expression in female mouse genital tracts. *Endocrinology* **125** 2888–2896.
- Zhao HH, Herrera RE, Coronado-Heinsohn E, Yang MC, Ludes-Meyers JH, Seybold-Tilson KJ, Nawaz Z, Yee D, Barr FG, Diab SG *et al.* 2001 Forkhead homologue in rhabdomyosarcoma function as a bifunctional nuclear receptor-interacting protein with both coactivator and corepressor functions. *Journal of Biological Chemistry* **30** 27907–27912.

Received 26 September 2003

Accepted 27 February 2004