Differential Expression of Claudin Family Proteins in Mouse Ovarian Serous Papillary Epithelial Adenoma in Aging FSH Receptor–Deficient Mutants

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Abstract
Ovarian cancer is a deadly disease with long latency. To understand the consequences of loss of follicle-stimulating hormone receptor (FSH-R) signaling and to explore why the atrophic and anovulatory ovaries of follicitropin receptor knockout (FORKO) mice develop different types of ovarian tumors, including serous papillary epithelial adenoma later in life, we used mRNA expression profiling to gain a comprehensive view of misregulated genes. Using real-time quantitative reverse transcription–polymerase chain reaction, protein analysis, and cellular localization, we show, for the first time, in vivo evidence that, in the absence of FSH-R signaling, claudin-3, claudin-4, and claudin-11 are selectively upregulated, whereas claudin-1 decreases in ovarian surface epithelium and tumors in comparison to wild type. In vitro experiments using a mouse ovarian surface epithelial cell line derived from wild-type females reveal direct hormonal influence on claudin proteins. Although recent studies suggest that cell junction proteins are differentially expressed in ovarian tumors in women, the etiology of such changes remains unclear. Our results suggest an altered hormonal environment resulting from FSH-R loss as a cause of early changes in tight junction proteins that predispose the ovary to late-onset tumors that occur with aging. More importantly, this study identifies claudin-11 overexpression in mouse ovarian serous cystadenoma.

Neoplasia (2006) 8, 984–994

Keywords: FSH receptor, tight junctions, ovary, aging, tumors.

Introduction
Ovarian cancer is a silent killer that is the most deadly of all gynecologic cancers striking women in their postmenopausal years [1], and the mechanisms that give rise to the late onset of this disease remain poorly understood. It is believed that the more frequent a woman ovulates during her reproductive life, the higher is her risk for epithelial ovarian tumors in later years [2]. Although premenopausal women develop more germ cell tumors with a lower incidence of epithelial tumors [3], cancer of the latter cell type predominates in postmenopausal women who suffer from ovarian cancer [4]. However, as not all postmenopausal women will develop ovarian cancer, other factors, including genetic predisposition or environment-related epigenetic influences, could increase the risk for ovarian tumors. Cellular and biochemical bases for differences in tumor types with aging, as well as initial processes that cause late tumorigenesis, are unknown. Clearly, a better understanding of these pathways will be helpful and crucial in developing early markers for disease prediction or detection. Although several cohort studies could not find an increased risk of infertility treatment for ovarian cancer development, data from a pooled analysis of case–control studies now suggest that infertility itself poses a significant risk [5]. Thus, the conundrum of ovulation risks and high circulating gonadotropins in menopause that propel and propagate epithelial tumors remains an enigma [6].

As ovarian epithelial tumors are rare in rodents, investigators have resorted to transgenic approaches directing viral antigens to compartments that can model ovarian cancers in mice [7], but these do not necessarily replicate hormonal imbalances that could be present in women or a menopausal environment where disease incidence remains highest. We have previously reported that follitropin receptor knockout (FORKO) mice mimic menopausal conditions in women [8] in that they exhibit hypergonadotropic hypogonadism, fail to ovulate, and remain sterile but develop ovarian tumors with aging [9]. Our recent microarray comparisons and other studies of mutant ovaries revealed early histologic changes in the ovarian surface epithelium (OSE) of null mutants from postnatal day 2 onward, escalating up to 12 months [10]. Migration of cells expressing cytokeratin, an epithelial cell marker, that began in 3- to 4-week-old female mice intensified with age, suggesting potential structural alterations within ovarian cells. By 12 months,
most animals developed tumors that could be classified into several categories, including granulosa cell tumors, Sertoli-Leydig cell tumors [9], and serous papillary epithelial adenomas (Figure 1) [10]. Identification of serous papillary epithelial adenoma was confirmed by lack of staining with 3\(\beta\)-hydroxy steroid dehydrogenase and inhibin-\(\alpha\). Such tumors were also stained by Ep-CAM and histologically resembled serous papillary adenoma found in human ovaries. Evidence of infiltration of epithelial cells or dedifferentiation within the ovary with increasing age indicated that neither incessant (multiple) ovulation nor the presence of follicle-stimulating hormone receptor (FSH-R) on the OSE is required to induce the migration of epithelial cells in a mutant ovary.

Several recent studies describing altered expressions of tight junction (TJ) proteins in epithelial ovarian tumors in women hold such alterations accountable for epithelial cell growth and survival [11–14]. As these studies are confined to the use of late tumor tissues or established cells derived therefrom, there is a critical gap in knowledge on the etiology or chronology of changes in proteins of normal ovarian surface epithelial cell junctions and their propensity to neoplastic transformations under altered conditions that could arise as part of pathology or normal aging. Studying the ovaries of FORKO mice at an age before the appearance of pathology, we now show for the first time that hormonal imbalances play an important role in inducing alterations in four ovarian claudin family member proteins that are known to act as proto-oncogenes. We provide evidence for their exclusive cellular localization in ovarian surface epithelial cells of FORKO mutants and in serous papillary epithelial adenoma. Additional studies with cells also indicate a direct role for hormones in epithelial cells. These observations might provide the foundation for exploring mechanisms and signaling pathways, as well as for testing novel options for reversal.

Materials and Methods

Animals and Tissue Collection

The studies described in this report were performed according to accepted and approved guidelines of the institutional animal care committee. FORKO mice were established...
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as described [8] by breeding 129T2/SV EmsJ FSH-R+/− male and female mice aged 3 to 5 months. These breeding pairs provided littermate +/+ and −/− females that would allow direct comparison. All mice were fed standard laboratory chow (5001/Harlan Teklad S-2335 diet, 1:1 mixture; Harlan Teklad, Madison, WI) and were maintained under a 12L:12D regimen.

**Affymetrix Gene Chip mRNA Expression Analyses and Quantitative Polymerase Chain Reaction (Q-PCR)**

We first hypothesized that direct microarray analysis comparing ovaries from normal and mutant mice would provide clues to global changes in gene alterations. For microarray analysis, ovaries from age-matched 8-month-old wild-type or FORKO mice (n = 3) were used. This age was chosen because we wanted to examine the latency of ovarian gene changes before overt tumors developed. The Affymetrix 39K GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) with 45,000 probe sets analyzes > 39,000 transcripts and variants of mouse genes. GeneChip arrays were used according to Affymetrix protocols (Affymetrix). Comparison of data between wild-type and FORKO ovaries was performed with GeneSpring 7.2 software (Silicon Genetics, Redwood City, CA) with default normalization scheme.

For RNA extraction in Q-PCR, both ovaries from a different set of animals (n = 5 for wild type and FORKO) were carefully dissected and pooled. Wild-type mice were selected at random without regard to the stage of estrous cycles, and this characteristic does not apply to mutants as they do not cycle. Q-PCR was performed using Quantitect Probe Q-PCR Kit (Qiagen, Mississauga, Ontario, Canada). Ovaries of different transcripts were determined in relation to β-actin with real-time PCR on Mx4000 (Stratagene, La Jolla, CA).

**Ovarian Gene Expression in Normal Mice Treated with Gonadotropin**

To investigate if claudin gene products relevant to this report and identified as differentially regulated by microarray were the result of lack of FSH-R signaling, we injected sexually immature 24-day-old wild-type mice and age-matched FORKO mice with 5 IU of equine chorionic gonadotropin (eCG; a FSH surrogate hormone) and performed Q-PCR analyses on ovarian RNA 24 hours posttreatment. This treatment synchronizes immature follicles in wild type by accelerating their development and by allowing rapid analysis of hormonal effects. Age-matched untreated wild-type and FORKO mice were used as controls (n = 5). Ovaries were collected for RNA processing as above.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)–Western Blot Analysis**

Whole ovaries from 8-month age-matched wild-type and FORKO mice (n = 6) were used for protein isolation using a standard lysis buffer containing protease inhibitors. Forty micrograms of total protein was separated by SDS-PAGE. After transfer, membranes were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal antibody to claudin-1 (1:200; Zymed Laboratories, San Francisco, CA), claudin-3, claudin-4, and claudin-11 (1:200; a kind gift from Dr. M. Furuse, Kyoto University, Kyoto, Japan). Alkaline phosphatase–conjugated secondary antibodies (1:10,000) diluted in blocking reagent were incubated with membranes for 1 hour at room temperature. Immunoreactive protein was visualized using chemiluminescence (Bio/Can Scientific, Inc., Mississauga, Ontario, Canada), and band densities were computed for comparison.

**Immunofluorescence**

Fresh frozen ovarian sections of 8-month-old mice (wild type and FORKO) and serous papillary epithelial adenoma samples from 1-year-old null mice were cryosectioned and fixed in acetone for 15 minutes, air dried in room temperature, and frozen at −20°C until analysis. Primary antibodies were used at 1:100 concentrations for claudin-1, claudin-3, claudin-4, and claudin-11, and fluorescein isothiocyanate–conjugated secondary antibodies (Sigma, Ontario, Canada) were used at the recommended concentration of 1:120. To quantify fluorescence intensities, the histology of samples was evaluated with a cursor drawing delineating the region of interest, which is called mask. That region/mask was then applied to the corresponding fluorescent image, and area fluorescence intensity was measured. Using constant settings, 100 epithelial cells selected at random from five different wild-type and FORKO mice were electronically scanned with 512 × 512 pixels. The images were recorded with 8 bits/pixel, resulting in 255 intensity levels. Using the LSM software (LSM 510, ApoChromat x40; Zeiss, Jena, Germany), the intensity of immunofluorescence above the threshold was measured (mean intensity per pixel).

**Culture of ID-8 Ovarian Epithelial Cells**

In the absence of an established mouse ovarian surface epithelial cell line (MOSEC) from FORKO mutants, we resorted to an epithelial cell line from normal mice (ID-8) that was kindly provided by Drs. Paul F. Terranova and Katherine Roby (University of Kansas Medical Center, Kansas City, KS) to investigate the impact of hormones. These cells, which were obtained after serial passage and have the potential to induce peritoneal tumors in recipient mice, were grown in plastic culture ware, as described previously [15]. Cells (5000–100,000 per well) grown for 2 days in six-well plates were starved of serum and growth factors overnight before treatment with different hormones, namely, highly purified hFSH (1–200 ng/ml) and hLH (1–200 ng/ml) (both from our laboratory), 17β estradiol (10⁻⁶–10⁻⁹ M), testosterone (10⁻⁶–10⁻⁸ M), and progesterone (10⁻⁶–10⁻⁹ M) (all from Sigma, St. Louis, MO) for 24 hours. Protein hormones were dissolved in saline, and steroid hormone stock solutions were prepared in ethanol and diluted with culture medium. Cells were lysed following treatment, and claudin protein expression was quantified by Western blot analysis using the respective antibodies. Actin determined for each well and in the same blot was used for normalizing and comparing untreated wells.
Steroid Hormone Levels and Statistical Analysis

Plasma levels of steroid hormones at two different ages (pretumor, 3 months; posttumor, 12 months) were measured using specific radioimmunoassays as described earlier [16]. Statistical differences were assessed by Student’s t test and considered significant at P < .05. Data in various figures are presented as mean ± SEM.

Results

Affymetrix Profiling of mRNA Expression in FORKO Ovary

Aging FORKO mice exhibit varied ovarian pathologies, with tumors developing in the majority of females by 1 year [9], including serous papillary epithelial adenoma in 30% of aging mice (Figure 1). The epithelial nature of these tumors was confirmed by histologic examinations and expression of appropriate cell markers [10]; the description of their nature will be reported elsewhere (Chen et al., manuscript in preparation). These observations prompted the current investigation to first determine global gene changes using Affymetrix microarrays. We hypothesized that a global analysis of the ovary at an age before tumor development would indicate evidence of gene expression changes that predispose the ovary to autocrine and paracrine interactions among different and altered compartments within the ovary. Additional details of these analytical studies, including the classification of altered gene families, will be reported elsewhere (Aravindakshan et al., manuscript in preparation). The complete data are available through the Gene Expression Omnibus (GEO) data repository (http://www.ncbi.nlm.nih.gov/geo/; GEO accession number GSE43232). As this initial analysis provided a number of candidates, including those that could be novel to the ovary, we chose two sets of genes (platelet-derived growth factors [PDGFs] and their receptors, and the claudin family) for further exploration. Among other considerations was their relationship with the OSE. Our findings on alterations in PDGF ligands and receptors in normal ovaries and mutant ovaries, including expressions in surface epithelial cells, have been recently reported [17]. Genes of the claudin family discussed here, consisting of 23 members participating in TJs and involved in epithelial cell–cell interactions, have attracted increasing attention to ovarian cancers [18].

Genes Encoding TJs Are Altered in the Absence of FSH-R Signaling

Among numerous differentially expressed genes in mutants are claudin family genes that have not been well characterized. Four members of this large family of proteins proposed to be involved in TJs are expressed in the mouse ovary. Microarray data showed consistent high expression of claudin-11, followed by claudin-3 and claudin-4, and down-regulation of claudin-1 gene in mutant ovaries. Q-PCR analysis confirmed that although claudin-1 decreased 2.6-fold (Figure 2A), claudin-3, claudin-4, and claudin-11 increased 2.9-fold (Figure 2B), 2.4-fold (Figure 2C), and 10.2-fold (Figure 2D), respectively, in 8-month-old FORKO ovaries when compared with age-matched wild-type mice. To explore how early these changes might begin following FSH-R deletion, we again compared ovarian claudin gene expression in age-matched 24-day-old wild-type and FORKO mice by Q-PCR. In young FORKO mice, claudin-1, claudin-3, and claudin-11 were altered as early as 24 days, whereas claudin-4 did not change (data not shown). This strongly suggests that claudin-1, claudin-3, and claudin-11 gene changes are among the very early events of impending cellular alterations, whereas claudin-4 might be considered as a marker of late events. The importance of these gene changes to considerations of ovarian epithelial cells will become evident in the following sections. The relevance of these sequential observations that occur in the absence of FSH-R signaling is important to note because delineation of such a chronology of events is possible only in defined models, age comparisons are impractical in women, and most expression studies are performed in late-stage ovarian cancers.

Comparison of Ovarian Gene Expression in Mice Treated with eCG

To test the hypothesis that changes in claudins in FORKO ovaries are induced by lack of FSH signaling, we performed a corollary experiment in which 24-day-old wild-type mice were stimulated for 24 hours by eCG, an FSH-R agonist that induces ovarian stimulation regardless of endogenous FSH. FORKO mice of the same age were also treated as part of this experiment for comparison. In wild-type females, claudin-1 mRNA was not altered, but expression of claudin-3 and claudin-4 showed a decreasing trend following eCG treatment (Figure 2, A–C). This treatment in wild-type mice also significantly downregulated ovarian claudin-11 mRNA levels (Figure 2D) and was consistent with the opposite effect seen in FSH-R mutant ovaries. No change in claudin mRNA levels was seen in FORKO mice treated with eCG, as they did not respond to the deletion of FSH-R. As FSH signaling in normal ovaries initiates a number of changes, including increase in hormonal steroids, this suggests that altered hormones, such as increased luteinizing hormone (LH) and androgen or decreased estrogen and progesterone levels in FORKO mice [16] (Figure 3, A and B), might play a role either individually or synergistically to alter the expression of claudin members. At present, virtually nothing is known on the impact of steroid hormones in the regulation of claudin genes in the ovary or in tumors (in animals or humans).

Ovarian Protein Expression Patterns in Mutants

To determine if transcriptional changes noted by altered mRNA in mutant ovaries are also reflected at the translational level, we quantified protein expression using Western blot analysis. Our immunoblot results using specific antibodies demonstrated that the amount of claudin-3, claudin-4, and claudin-11 proteins was significantly increased in mutants at 8 months, whereas claudin-1 was drastically reduced or undetectable in most experiments (Figure 4, A–D). In addition to revealing claudin-11 protein expression in the mouse ovary for the first time, the results also show that, among the four claudin proteins compared between wild-type and mutant
mice, claudin-11 expression showed the highest change (a 250% increase). This trend is consistent with the changes in mRNA noted above. As change in ovarian claudin-11 gene/protein was most predominant, we used it as a representative of this family, focusing attention on its ontogeny in mutants and wild-type mice. Western blot analysis carried out in age-matched, wild-type, and FORKO mice at 24 days, 3 months, 6 months, and 12 months revealed a significant and sustained increase in claudin-11 in mutants when compared to that in wild-type mice (Figure 4E). Claudin-11 protein levels in wild-type mice remained constant. These results confirm the above findings (based on gene expression and mRNA levels) that selected claudins are altered in the ovaries of FSH-R knockout mice. Cellular localization within the ovary, as noted below, corroborates these observations and provides further evidence for selective expression and changes in surface epithelial cells of mutants.

**Immunofluorescence—Localization and Cellular Changes**

To discount the possibility that different components of the ovary might have contributed to differences seen in total protein expression, we used comparative imaging and quantitation with confocal imaging systems. Using constant settings, 100 epithelial cells selected at random from different wild-type ovaries, FORKO ovaries, and tumor samples were scanned for immunofluorescence (Figure 5), confirming the above findings of Western blot analysis. Compared with +/- animals, FORKO mice exhibit epithelial proliferation with
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Figure 3. Circulating sex steroid hormones and aging in female mice. (A) Plasma levels of steroid hormones were measured by specific radioimmunoassays at 3 and 12 months of age. Testosterone (n = 11–15 mice) and estradiol (n = 12) ratios are shown depicting androgen dominance in FORKOs. (B) Plasma levels of progesterone (ng/ml) measured at 3 and 12 months (n = 8). Asterisks indicate significant differences from wild-type mice (P < .05).

stratification, as seen in more than one layer of epithelial cells by 8 months of age and in epithelial tumors developing by 12 months of age (Figure 5). It is notable that the TJ-associated proteins claudin-1, claudin-3, claudin-4, and claudin-11 were distributed exclusively along epithelial cell borders in +/+ mice (Figure 5, A–D). Their exclusive localization to ovarian epithelial cells, but not to either granulosa cells or thecal cells, which are the classic dominant targets of gonadotropins in the ovary, suggests a potential role in maintaining cell integrity and barriers to epithelial cell migration under normal circumstances. In FORKO mice and epithelial adenomas, staining was increased both in the cytoplasm and along epithelial cell borders (Figure 5, E–L). This first report of claudin-11 expression, a suggested proto-oncogene [19], on the surface of mouse ovarian epithelial cells (Figure 5D) and its increase in the absence of FSH-R signaling (Figure 5, H and L) signify important cellular and structural changes in the epithelium long before tumor propagation occurs. In negative controls, no staining was seen. Its aberrant cellular localization, in part, could also be significant in altering cell–cell interactions and signaling. Other tumor types that also appear in FORKO ovaries [9] were not stained by antibodies to claudins (data not shown), indicating that upregulation of these proteins as found in this study is specific to the OSE type of tumors. Quantitative immunofluorescence showed that, in FORKO ovarian samples, claudin-3, claudin-4, and claudin-11 content was significantly increased (P < .05) compared to ovarian epithelial cells in +/+ mice. However, the greatest increase was again seen in claudin-11 protein expression. Interestingly, faint or very little staining was observed for claudin-1, confirming data in Figures 3 and 4. These data reinforce the idea that selected claudin proteins participating in cell–cell interactions and functioning as potential oncoproteins are differentially expressed in epithelial cells of FORKO ovary.

Hormonal Regulation of Adhering and TJ Proteins in Cultured Epithelial Cells

Hormonal imbalances that are of significance to diverse pathology occur early in life in FORKO mutants, as depicted by circulating levels (Figure 3). Of particular interest to this report are aberrations shown as testosterone/estrogen ratio and decreased progesterone that occurs early and is sustained with aging. As powerful hormonal transcriptional regulators, it is highly likely that these hormonal changes could alter nuclear receptor interaction in cells. Although epithelial cells isolated from FORKO mice at different ages would constitute ideal systems for comparison with similar cells from normal mice and for identifying aberrations and mechanisms, such cells are not yet currently available for our study. As an alternative to this and to determine if components contributing to hormonal imbalances in FORKO mice could alter TJ proteins in the ovarian epithelium, we treated an available mouse OSE cell line (ID-8) individually with five hormones (FSH, LH, 17β estradiol, testosterone, and progesterone) for 24 hours. As indicated in the Materials and Methods section, this cell line was established by serial passage of OSE derived from normal mice [15]. This question becomes relevant because, in addition to high FSH and LH [16], FORKO mice have sex steroid imbalances (Figure 3, A and B), indicating androgen dominance and progesterone deficit. We anticipated that such a study would provide information relative to possible direct influences, if any, on these types of cells. For brevity and clarity, results chosen from the effects of two concentrations are summarized in Figure 6. Treatment with FSH induced a significant decrease in claudin-11. Although LH increased claudin-3, testosterone increased claudin-3, claudin-4, and claudin-11 levels. There was no effect on the low expression of claudin-1 by any of the hormones tested in this cell line. The effect of androgen on this cell line derived from the wild-type mouse epithelium is particularly noteworthy as it is considered a risk factor for ovarian tumors in women [20]. In support of this, we have found that, among the hormones tested, only androgen induced the maximal migration in ID-8 cells in culture (data not shown). Estradiol showed a trend in increasing claudin-4 levels. Interestingly, progesterone did not affect any of the proteins examined, suggesting that the apparent beneficial effects of progesterone in suppressing ovarian tumors in women [20,21] could be mediated by other pathway(s) or
by interactions with other compartments in the intact ovary. To our knowledge, this is the first study to show that different hormones produced by the ovary and known to participate in paracrine and intracrine actions can also directly and acutely alter normal mouse ovarian epithelial cell junction proteins and also occur exclusively in tumors of this cell type.

**Discussion**

Gonadotropins as important regulators of ovarian function act by binding to specific receptors in target cells. Our recent studies of FSH-R mutant ovaries revealed early histologic changes in the OSE of null mutants from postnatal day 2 onward and escalating up to 12 months [10]. By 12 months,

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**Figure 4.** Evidence for quantitative alterations in proteins associated with maintaining cell polarity in the ovary. A representative Western blot image of (A) claudin-1, (B) claudin-3, (C) claudin-4, and (D) claudin-11 in 8-month-old ovaries of wild-type and FORKO mice (before tumors appear) is shown. (E) To further understand the ontogeny of claudin-11 expression, claudin-11 expression was compared in age-matched, wild-type, and FORKO mice at 24 days, 3 months, 6 months, and 12 months. A representative image is shown here. Lanes 1, 3, 5, and 7 are protein extracts from wild-type mice. Lanes 2, 4, 6, and 8 are protein extracts from FORKO mice. Protein levels were determined by densitometry and corrected for protein loading using β-actin levels. Data are expressed as the percentage of wild-type expression from three different experiments. Asterisks indicate significant differences from wild-type mice (P < .05).
most animals develop tumors that can be classified into several categories, including granulosa cell tumor, Sertoli-Leydig cell tumor [9], and serous papillary epithelial adenoma [10]. In support of this, studies from another laboratory, using an independently derived FSH-R knockout or its ligand (FSH) knockout (FSHbKO), resulted in the development of serous ovarian adenomas in aging mice [22]. Because FSH action is absent in FORKO females due to lack of FSH-R, chronic exposure to high concentrations of LH and androgen would appear to be the primary cause of the observed pathology. In transgenic mice overexpressing LH transgene [23], increased plasma LH concentrations have been implicated in the initiation of subsequent ovarian pathology, but no information on the role or alteration of TJ proteins in this model is available.

Most epithelial cells are characterized by the ability to form TJs. TJs, together with adherens junctions, form the apical junctional complex in epithelial cells. Adherens junctions are responsible for mechanical adhesion between adjacent cells, whereas TJs are essential for controlling paracellular ion flux and, therefore, for maintaining tissue homeostasis [24]. TJs also play a crucial role in the maintenance of cell polarity. Finally, because of the ability of TJ proteins to recruit signaling proteins [25], TJs have also been hypothesized to be involved in the regulation of proliferation, differentiation, and other cellular functions.

As our microarray studies indicated changes in these genes, we hypothesized links to ovarian pathology. We have investigated the expression and localization of the TJ proteins claudin-1, claudin-3, claudin-4, and claudin-11 in normal and...
Recently, it has become apparent that claudin gene expression is frequently altered in several human cancers. However, the exact pattern of claudin expression in different tumors is unknown, as only a limited number of claudin genes have been investigated in a few tumors. In fact, studies published thus far report an overexpression of claudin-3 and claudin-4 in late ovarian cancers [11–14]. In this study, we found that although there were significant increases in claudin-3 and claudin-4, the major increase was in claudin-11 expression. This might be due to differences in species or in the nature of the tumors studied. Rangel et al. [13] reported that, in the human ovary, claudin-3 and claudin-4 mRNA are expressed at low levels in the normal ovary, but are highly upregulated in epithelial ovarian cancers of all subtypes. Also by immunohistochemical analyses, it was confirmed that although a high level of expression of claudin-3 and claudin-4 occurs in the majority of ovarian carcinomas, ovarian cystadenoma did not frequently overexpress these proteins. In our mutant ovaries, most of the epithelial tumors are of the cystadenoma type. This suggests that overexpression of the TJ proteins depends on the histologic type of cellular alterations. In further support of these data, a review of the large publicly available SAGE database [26], which is used to ascertain the gene expression of claudin-11 in both normal and neoplastic human tissues, has revealed that claudin-11 was specifically overexpressed in ovarian cystadenoma (Table 1).

It is striking that the increase in claudin-3 and claudin-11 occurred long before tumors became apparent in our mutant ovaries. This would suggest that changes in claudin might trigger the increased epithelial cell migration that we see in mutant ovaries when compared to wild-type ovaries. This would appear to be consistent with increased claudin-3 and claudin-4 expression contributing to increased ovarian surface epithelial cell migration and survival [27]. Interestingly, claudin-11 has also been shown to modulate the proliferation and migration of oligodendrocytes [19]. Thus, future studies will take this into consideration when assessing the significance of increased claudin-11 expression in mutant ovaries and in the generation of serous papillary adenomas.

Previously, claudin-1 has been shown to be present in human ovarian surface epithelial cells and to be localized to cell borders. Claudins were shown to form functional TJs [28]. Decreases in claudin-1 expression in breast cancer [29,30], as well as in colon cancer [31], are suggestive of a possible relationship with either tumorigenesis or propagation. Similarly, the decrease in claudin-1 that we have observed in FORKO ovaries and, more particularly, in OSE, as well as in tumors attributable to this cell type in mutants (Figures 2 and 5), also indicates a close relationship with these two processes. It is believed that the exact combination of claudin

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**Table 1. SAGE Analysis of Claudin-11 Gene Expression in Normal and Neoplastic Ovarian SAGE Libraries.**

<table>
<thead>
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<th>GAPDH</th>
<th>Claudin-11</th>
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These data are derived from public databases. GAPDH levels are used as controls. Note that claudin-11 expression is higher in an ovarian cystadenoma library.
proteins within a given tissue can determine the selectivity and strength of TJs [32]. Therefore, in such cases, compromising TJ integrity due to loss of some claudin proteins and gain of other claudin proteins may allow inappropriate diffusion of paracrine factors, which may mediate the survival and growth of tumor cells. Our studies define, for the first time, the pattern of claudin expression and distribution in normal and tumorigenic mouse ovaries. In this study, we were not able to determine the hormonal regulation of claudin-1 as the presently available ID-8 cell line did not express claudin-1. We speculate that the serial passages [15] used for securing these tumorigenic cells, albeit from wild-type mice, might have led to the maximum downregulation of claudin-1 that could not be altered any further by additional treatments, as used in this study. Thus, it would be reasonable to propose that strategies directed at manipulating—in this case restoring—claudin-1 might prevent tumor cell propagation. 

Pituitary gonadotropins, androgens, estrogens, and progesterone, as well as insulin-like growth factor-1/2, have all been proposed to influence ovarian tumor development [4]. However, the role played by reproductive hormones on TJs of the ovary and on tumorigenesis is not known. This study, using a mouse model, shows that increased androgen in the absence of FSH-R signaling induces major perturbations in hormones that alter signal transduction pathway(s) and the internal milieu of the ovary, leading to alterations in OSE in mutants. Although we have not investigated different pathways, signaling through TGF-β, Wnt, and MAPK, which have been previously shown to be involved in regulating various cell junctions, is also depicted. Here we implicate that hormonal imbalances result in increases in claudin-3, claudin-4, and claudin-11, whose effects are transmitted downstream, causing ovarian surface epithelial cells to acquire the capacity to survive and migrate inside the ovary. Subsequently, serous papillary epithelial adenomas develop in aging FORKO mice.

Further evidence that epithelial cells in knockout mice are altered comes from the fact that there is an increased expression of E-cadherin and N-cadherin in our FORKO mutant ovaries (unpublished results). Unlike other epithelial tumors, E-cadherin (a tumor suppressor) and N-cadherin are overexpressed in epithelial ovarian tumors, enhancing cell proliferation and survival [33,34]. In summary, we propose that FSH-R signaling provides a balanced hormonal environment beneficial for regulating cell–cell interactions that organize and maintain polarity in surface epithelial cells of the ovary that exert restraining influence (Figure 7). Based on chronological evidence gathered from FORKO mice, we suggest that ovarian hormonal imbalances associated with (gradual) loss of FSH-R signaling in perimenopause and menopause, along with other genetic dispositions and environmental factors, might lead to aberrant expression of claudin family (and cadherin proteins), allowing inappropriate paracrine/intracrine influences emanating from other ovarian compartments to enhance cell migration and tumorigenic propensity. Understanding how events associated with early and altered expressions of selected claudin members and their signaling functions contribute to ovarian pathology will be critical. Further efforts to secure altered epithelial cells from FORKO mutants might accelerate in vitro and in vivo modeling studies aimed at understanding mechanistic issues and devising appropriate strategies aimed at reversal. Finally, our study confirms previously known claudin gene expression.
patterns in an experimental model and identifies major alterations in a new claudin member (claudin-11), which may have applications in the detection of ovarian cystadenomas. This work might help stimulate additional studies in exploring this possibility.

Acknowledgements
We thank M. Furuse for providing the claudin antibodies, and K. Roby and P. F. Terranova for giving us the ID-8 cells.

References


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References


