Tight Junction in Ovarian Surface Epithelium and Epithelial Ovarian Tumors

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To my family

献给我的家人

To most people solutions mean finding the answers. But to chemists solutions are things that are still all mixed up.

----- A deep thought from a child

ABSTRACT

Zhu, Yihong. 2007. **Tight Junction in Ovarian Surface Epithelium and Epithelial Ovarian Tumors.** Department of Obstetrics and Gynecology, Sahlgrenska Academy at Göteborg University, Sahlgrenska University Hospital SE-413 45 Göteborg, Sweden.

Epithelial ovarian cancer originating from ovarian surface epithelium (OSE) is the most lethal type of gynecological cancer among women worldwide. The poor understanding of the cellular and molecular events associated with ovarian carcinogenesis leads to difficulties in early diagnosis and in efficient treatment. Recently, much evidence has implicated that tight junction (TJ) could play a role in signaling pathways that regulate cell proliferation, polarization, and differentiation. Moreover, altered expression of TJ proteins have been discovered in many types of human epithelial tumors.

The general aims of this thesis were to investigate the expression, localization, function and modulation of TJ in normal OSE and epithelial ovarian tumors (EOT). Moreover, a further understanding of the possible roles of TJ in transformation of OSE towards EOT and in tumor progression was sought.

The studies were approved by the human Ethics committee of Sahlgrenska Academy, Göteborg University. Informed written consent was obtained from all women participating in the study.

Cultured OSE, EOT biopsies and cell lines were used in the studies. Formation of TJ was investigated by electron microscopy observation, immunofluorescence and western blot with semi-quantitative densitometry analysis. Ion-barrier function of TJ was evaluated by trans-epithelial resistance (TER) measurement. The results showed that: ***1.*** TJ proteins ZO-1, occludin and claudin-1 are expressed in normal OSE cells *in situ* and *in vitro*. TJ structure was confirmed by electron microscopy observation in early passage of cultured OSE. During culture of normal OSE, a low TER value was built up and could be interfered with by a Ca^{2+} chelator. ***2***. Claudin-3 and -4 were *de novo* expressed or up-regulated in ovarian epithelial inclusion cysts and EOT compared with normal OSE. Moreover, in ovarian serous and mucinous tumors, claudin-4 was significantly increased in borderline-type tumors and adenocarcinomas compared with benign tumors. Claudin-3 was significantly increased in adenocarcinomas compared with borderline-type and benign tumors; whereas no changes were found for claudin-1 or -5. ***3.*** In the study of four ovarian cancer cell lines, ZO-1, claudin-1, -3, -4 and E-cadherin were found to be expressed along the entire cells periphery in serous adenocarcinoma cells concomitant with high TER value, while clear-cell and endometrioid adenocarcinoma cell lines did not express claudin-4 and E-cadherin, concomitant with minimal TER values. ***4.*** When transforming growth factor (TGF)-β1 was added to cultured OSE and OVCAR-3 ovarian cancer cell line, the expression levels of TJ and adherens junction (AJ) proteins and TER values were changed. Furthermore, treatment with TGF-β1 induced an EMT-like morphological change in cultured OSE.

It is concluded that normal OSE forms TJ with a weak ion-barrier function. The TJ proteins claudin-3 and -4 are up-regulated in EOT. Specific function of TJ might depend on and differ in between various histological subtypes of ovarian cancer. TGF-β1 can modulate the formation of TJ and AJ, and the ion-barrier function of TJ in both OSE and epithelial ovarian cancer cells in culture. These findings suggest a potential role of TGF-β1 in epithelial ovarian tumorigenesis.

Key words: tight junction, claudin, ovarian surface epithelium, epithelial ovarian tumor, TGF-β1

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中文摘要：

起源于卵巢上皮细胞的卵巢上皮细胞癌是恶性度最高的妇科肿瘤。目前关于卵巢癌发病机理的认识仍然非常有限，因而限制了该肿瘤的早期发现和有效的治疗。紧密联结(tight junction)是上皮细胞的特征性结构之一，它是由多种蛋白结合而成的细胞间的连接结构。许多研究表明，该结构在人体组织中不仅能够调节上皮细胞层分子离子通透性，而且还能够调节和肿瘤发生发展密切相关的细胞增殖和细胞分化。 在许多人类肿瘤中，紧密联结的蛋白构成均被发现有明显的改变。本论文研究目的在于观察在卵巢上皮肿瘤细胞和正常的人卵巢上皮细胞中，紧密联结的蛋白构成，功能，及其调节因素。从而，进一步了解紧密联结在卵巢肿瘤起源和发展过程中所起的作用。

通过对人体样本细胞的直接观察和体外培养细胞的研究，我们发现，虽然正常人体卵巢上皮细胞和卵巢上皮肿瘤细胞都具有功能性的紧密联结结构，但是表现出了不同的蛋白构成形式，即两种紧密联结的膜蛋白(claudin-3, claudin-4)仅在肿瘤细胞中被发现，而在正常细胞中通常缺失，而且这两种蛋白的表达随肿瘤的恶性度增加而上调。此外，我们还发现人类转化生长因子(transforming growth factor)可以在体外逆向调节上述两种膜蛋白的表达。

这两种膜蛋白同时又被其他学者发现是产气荚膜梭菌肠毒素的膜受体，当该毒素结合到受体上，细胞膜被毒素穿透而破坏，引起其细胞内渗透压的改变，而最终导致细胞死亡。我们的发现提供了利用产气荚膜梭菌肠毒素治疗卵巢癌的可能性，同时也提供了两个卵巢上皮细胞肿瘤标记物。本研究也暗示了这两种膜蛋白的表达和卵巢癌发生具有特殊的相关性，同时转化生长因子在人类卵巢癌发生发展中可能具有的潜在的保护作用。

------谨致中国的家人和朋友

LIST OF PUBLICATIONS

This thesis is based upon the following papers, which will be referred to in the text by their Roman numerals:

I. **Formation and barrier function of tight junctions in human ovarian surface epithelium.**
Zhu Y, Maric J, Nilsson M, Brannstrom M, Janson PO, Sundfeldt K.
Biol Reprod. 2004 Jul;71(1):53-9.

II. **Differences in expression patterns of the tight junction proteins claudin 1, 3, 4 and 5, in human ovarian surface epithelium as compared to epithelia in inclusion cysts and epithelial ovarian tumors.**
Zhu Y, Brannstrom M, Janson PO, Sundfeldt K.
Int J Cancer. 2006 Apr 15;118(8):1884-91.

III. **Tight junction formation and function in serous epithelial ovarian adenocarcinoma.**
Zhu Y, Sundfeldt K.
Manuscript.

IV. **TGF-β1 modulates tight junction and the expression of cadherins in cultured ovarian surface epithelium and epithelial ovarian cancer cells.**
Zhu Y, Nilsson M, Sundfeldt K
Manuscript.

TABLE OF CONTENTS

ABBREVIATIONS

AJ	Adherens Junction
AR	Androgen Receptor
ASIP	Agouti Signaling Protein
BRCA	Breast Cancer Gene
CA125	ovarian Cacinoma Antigen125
CK8	Cytokeratin8
CPE	Clostridium Perfringens Enterotoxin
CRB	Crumbs
DAB	3,3'-Diaminobenzidine
EGF	Epidermal Growth Factor
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-t etraacetic acid
EMT	Epithelio-Mesenchymal Transition
EOC	Epithelial Ovarian Cancer
EOT	Epthelial Ovarian Tumor
ER	Estrogen Receptor
FIGO	International Federation of Gynecology and Obstetrics
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GUK	Guanylate Kinase
hCG	human Chorionic Gonadotropin
IC	Inclusion Cysts
JAMs	Junctional Adhesion Molecules
LEF	Lymphoid Enhancer-binding Factor
LH	Luteinizing Hormone
MAGI	Membrane-Associated Guanylate kinase
MDCK	Madin-Darby Canine Kidney
MMPs	Matrix Metalloproteinases
MT1-MMP	Membrane Type Matrix Metalloprotease 1
OSE	Ovarian Surface Epithelium
PDZ domain	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zo-1 protein domain
PR	Progesteron Receptor
SH3 domain	Src homology-3 domain
siRNA	small interfering RNA
Smad	Sma and Mad related protein
TCF	T-Cell Factor
TER	Trans-Epithelial Resistance
TGF	Transforming Growth Factor
TJ	Tight Junction
TβRI	Transforming Growth Factor β receptor I
TβRII	Transforming Growth Factor β receptor II
VAMP	Vesicle-Associated Membrane Protein
VAP-33	Vesicle-associated membrane protein -Associated Protein-33
WHO	World Health Organization
ZAK	leucine-zipper (LZ) and sterile-alpha motif (SAM) kinase
ZO-1	Zonula Occluden 1
ZO-2	Zonula Occluden 2
ZO-3	Zonula Occluden 3
ZONAB	ZO-1-associated nucleic acid-binding protein

INTRODUCTION

1. Ovarian Surface Epithelium

General Introduction of OSE

Ovarian surface epithelium (OSE), which is also referred to in the literature as normal ovarian epithelium or ovarian mesothelium is a monolayered squamous-to-cuboidal epithelium. It is a continuation of the peritoneal mesothelium and covers the surface of ovary (1). The OSE is separated from the ovarian stroma by a basement membrane (basal lamina) and, underneath, by a dense collagenous connective tissue layer, the tunica albuginea (Figure 1). The cells of OSE are loosely adhered to the basal lamina and can easily be removed by scraping or brushing the surface of the ovary (2).

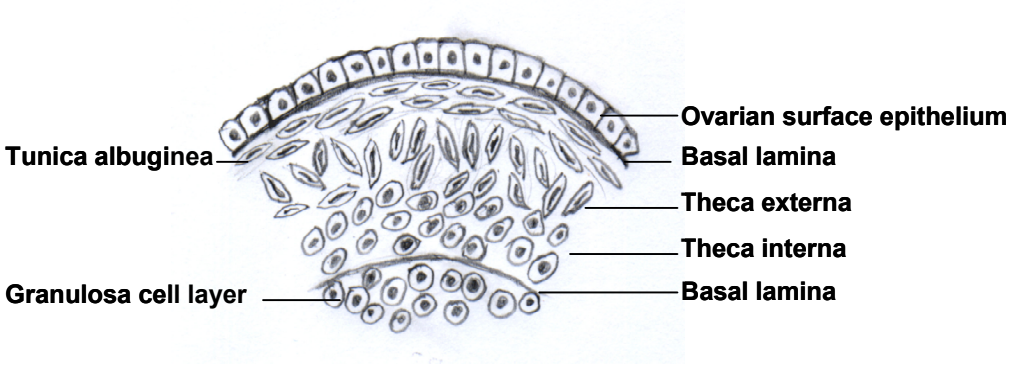

Figure 1. Schematic representation of preovulatory follicle wall of the human ovary.

In the postpubertal woman, normal stationary OSE has no known tissue-specific differentiation markers. *In situ,* it expresses keratin types 7, 8, 18 and 19, which represent the keratin complement typical for simple epithelia. The expression of mucin antigen MUC1, 17β-hydroxysteroid dehydrogenase and presence of cilia could distinguish it from extraovarian mesothelium (1). Intercellular contact and epithelial integrity of OSE are maintained by simple desmosomes, incomplete tight junctions (3), several integrins (4), and cadherins (5, 6).

Although the OSE represents only a diminutive fraction of the diverse cell types that comprise the ovary, it accounts for about 90% of all cases of ovarian cancer (7). This might be due to the multi-differential potential of OSE and its special physiological environment.

Embryonic Development and Multi-differential Potential of OSE

Early in embryonic development, the zygote is developed into a bilaminar embryo, with two germ layers (ectoderm and endoderm). Afterwards, some epithelia of the ectoderm layer transform into freely migrating mesenchymal cells (8) (Figure 2A), migrate between the endoderm and the ectoderm, and form the third germ layer, which is termed as intraembryonic mesoderm. The intraembryonic mesoderm further differentiates into three portions (Figure 2B): paraxial mesoderm, intermediate mesoderm and lateral mesoderm. While the lateral mesoderm splits into two layers (somatic lateral mesoderm and splanchnic lateral mesoderm), the space between these layers is formed as intraembryonic coelom. Meanwhile, the surrounding mesoderm layer derives into intraembryonic coelomic epithelium (mesothelium). The future OSE is formed from part of this mesodermally derived intraembryonic coelomic epithelium. This intraembryonic coelomic epithelium is also the precursor of the pleura, peritoneum, pericardium and Müllerian duct-derived epithelium, *e.g.* upper vagina, cervix, uterus and oviducts.

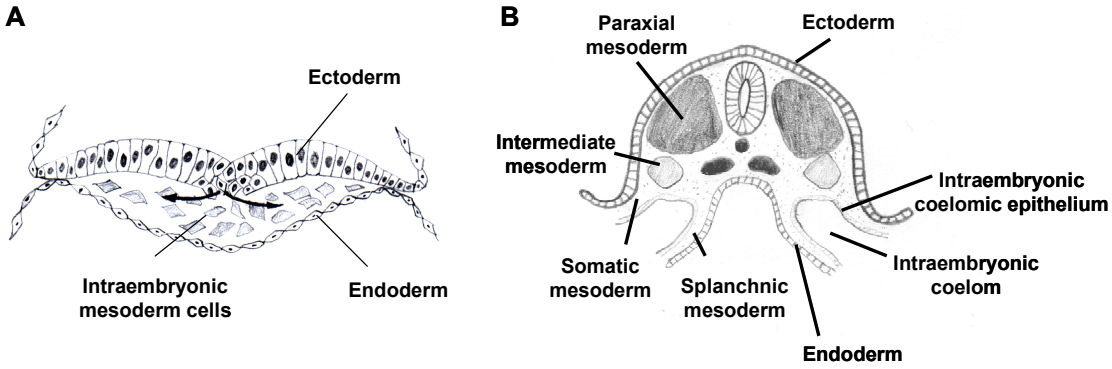

Figure 2. Schematic representation of early stages in human embryonic development. (A), The conversion of a bilaminar to a trilaminar embryonic disc (ectoderm, mesoderm, endoderm) involves a major epithelial/ mesenchymal transition. (B), The formation which has differentiated from the early intraembryonic mesoderm.

This embryonic process that is closely related to epithelial/mesenchymal interaction somehow indicates an intimate biologic relationship between epithelium and mesenchyme in mesodermal tissues. In fact, in the adult woman, all above mentioned coelomic epithelium-derived mesothelium retains a mixed epithelio/mesenchymal phenotype. For example, in these cells, the cytoskeleton contains not only the epithelial type of intermediate filament, keratin, but also vimentin, which is commonly found in mesenchymal cells (9) (Figure 9A).

Furthermore, OSE and extraovarian peritoneum have the capacity to secrete the stromal collagens of type I and type III (9).

During postovulatory repair, OSE adjacent to the ovulation rupture area has been found to modulate to a fibroblast-like form (10, 11) (Figure 9B). A similar transition was also showed in OSE (9, 12, 13) and other mesodermally derived epithelia (*e.g.,* kidney epithelium and endothelial cells) (14) with passages in culture. The response of the cells in explantation into culture may mimic their response to ovulatory rupture and other forms of injury, since explantation into culture could generally be assumed to be a kind of wound healing process (1). Therefore, it is indicated that epithelio-mesenchymal conversion is part of normal OSE physiology (1).

It has been reported that the epithelial differentiation marker CA-125 is expressed in the adult in extraovarian peritoneum and Müllerian epithelia, but not in the OSE, even from early stage in development (15). This difference could be an indication of the evidence of divergent differentiation between OSE and other mesothelium. In line with this part of the coelomic epithelium that gives rise to the OSE does not reach the stage of differentiation where CA125 is expressed as in other coelomic epithelial derivatives. This interpretation is in keeping with the concept that OSE is developmentally less mature than other mesothelium and that its development is arrested at a progenitor stage. Evidence that the growth potential of OSE is greater than that of extrovarian peritoneum (16) also supports this speculation.

In fact, many studies (6, 17-19) has demonstrated that OSE keeps its potential to further differentiate towards Müllerian-duct derived epithelium, which is thought to be mature formed mesothelium. When OSE transforms into neoplasm or inclusion cyst formation, it often expresses CA125 and E-cadherin *de novo* (5, 6, 20-22). E-cadherin has also been shown to be a differentiation marker for normal Müllerian epithelia (23). Thus, in contrast to epithelio-mesenchymal conversion, the differentiation of OSE towards Müllerian-duct derived epithelium is assumed as a pathophysiological process, which accompanies with metaplastic and/or neoplastic transformation of OSE (1) (Figure 9C).

OSE under Physiological Environment in Adult

In the adult, the OSE is believed to actively participate in the ovulatory process. It has been suggested that proteolytic enzymes released from cytoplasmic granules of epithelial cells degrade the tunica albuginea and underlying apical follicular wall, thereby weakening the ovarian surface to the point of rupture (24). Those OSE cells

located directly over the point of rupture undergo apoptotic cell death and are shed from the ovarian surface before ovulation (25). The wound created at the ovarian surface is repaired by rapid proliferation of OSE cells from the perimeter of the ruptured follicle (26). It has been described that the OSE on the ovulation sites acquired a flat squamous-like appearance, which was thought to be a metaplastic process in response to chronic surface injury at ovulation (27). The repeat of this process provides an opportunity for the accumulation of mutations that may contribute to the carcinogenesis.

The OSE is located near the source of hormones and growth factors, which is mostly produced by follicles/ corpus luteum within the ovary, and is exposed to some of them at high concentrations in a cyclic manner. Thus, OSE is more prone to be influenced than other types of mesothelium within the abdomen. OSE cells express receptors for estrogens (28-32), androgens (31, 33), progestins (30-32, 34), GnRH (35), FSH (36-38), LH (39, 40), and for growth factors, such as EGF, TGFα (41) and TGFβ (42-44). The effects of these agents on the physiology and pathology of OSE are incompletely defined and/or controversially discussed. However, some of them will be briefly summarized in the latter section (*Etiology of EOC*).

2. Inclusion Cysts

With age, epithelial inclusion cysts (IC) in the ovarian cortex are more frequently observed. It is widely believed that these IC arise from OSE (45), though the mechanism by which OSE transformed into IC is still controversial (45-47). Many lines of evidence including the finding that intraepithelial carcinomas and precarcinomatous lesion can be observed in IC (45) support the hypothesis that IC is the potential origin of epithelial ovarian tumor (EOT). IC has also been indicated to undergo metaplastic changes, *i.e.* to take on phenotypic characteristics of Müllerian epithelium. These characteristics including columnar cell shapes and expression of CA125 and E-cadherin are also found in ovarian neoplasms (6, 17, 20, 21). In addition, a study, which showed that inclusion cysts more frequently appeared in the ovaries of women with hereditary risk of ovarian cancer than in other women, also strengthens this hypothesis (48).

3. Epithelial Ovarian Tumor

Epidemiological and Clinical Aspects of EOT

Tumors of ovarian surface epithelial origin, which constitute about two-thirds of all ovarian neoplasms, are termed epithelial ovarian tumor (EOT) (49); within them, the malignant EOT is further named as epithelial ovarian cancer (EOC) or ovarian carcinoma. EOC is the fifth leading cause of all female cancer-related deaths in the western world, and it is the most prevalent and lethal of all gynecologic cancers. Approximately 60% of the women who develop ovarian cancer will die from their disease. Lack of an adequate screening test for early disease detection, coupled with rapid progression to chemoresistance, has prevented appreciable improvements in the five-year survival rate of patients with ovarian cancer.

According to the histological classification of ovarian tumors by the World Health Organization (WHO), EOT can be grouped into the following histological types: serous, mucinous, endometrioid, clear cell, transitional cell (Brenner) tumors, mixed epithelial, and other types. Among them, the first four types are most common. According to the pattern of invasiveness of the tumor cells, these four tumor types can be classified as benign, borderline and malignant tumor, individually.

The most commonly utilized staging classification system for EOC is the (International Federation of Gynecology and Obstetrics) FIGO system (50). It is based on findings of the size of the tumor, the extent of the tumor's growth into other tissues, whether the lymph nodes are involved, and the spread of cancer to other areas of the body (metastasis). The staging is done mainly through surgical exploration in combination with histological analysis. The EOCs are also histologically subclassified by grading (50), which refers the grade of differentiation by histological examination.

Histological classification, staging and grading diagnosis of EOC provide the most significant guideline for both treatment and prognosis (51-54). For example, in FIGO Annual Report, Vol. 26, 7314 cases of ovarian malignancy were collected, and the data analysis showed according to data in year 1990-2001, that stage and grade are major prognostic markers (51).

Etiology of EOC

Well-established risk factors for ovarian cancer are age (50-69 years) (55), family history of ovarian cancer, and infertility, whereas increasing parity, duration of lactation (56), oral contraceptive use (57, 58), hysterectomy (59, 60) or tubal ligation

decrease risk (56, 61, 62). These epidemiologic characteristics of ovarian cancer have given rise to several etiologic hypotheses, including the incessant ovulation hypothesis (63), the gonadotrophin hypothesis (64), the sex steroid hormonal hypothesis (61) and the inflammation hypothesis (65).

The most widely cited is the incessant ovulation hypothesis, proposed by Fathalla (63). The hypothesis states that repeated ovulation, with its successive rounds of surface rupture and OSE cell mitosis to repair the wound, renders the cells susceptible to malignant transformation. This hypothesis is supported by the facts that all the above mentioned factors, which concern the decrease of ovulation number in a lifetime (*i.e.* multiparity, lactation, oral contraceptive use, early menopause) substantially reduce the risk of ovarian cancer. When the total number of ovulations during lifetime was calculated for women who had EOC and for those that did not, a significant correlation between high total number of ovulation during lifetime and the occurrence of cancer was found (66). The fact that the only species other than humans to frequently develop EOC are hens, specifically those domestic hens that have been hyperovulated to produce eggs (67), adds a further support for the incessant ovulation hypothesis. Ovulation-induced DNA damage in ovarian surface epithelial cells at the periphery of the ovulatory site has been reported in sheep ovaries (68). Moreover, there are studies showing that primary cultures of normal rat and mouse OSE, which have been repeatedly subcultured to maintain continued proliferation, acquire features associated with malignant transformation, and ability to form tumors in nude mice (69, 70). These findings provide the evidence that OSE could undergo mutagenic transformation via frequent mitosis.

Gonadotropin levels increase with increasing age and are particularly high during menopause, consistent with the age-specific rates of EOC (71). This is the underlying base for the gonadotropin hypothesis. The gonadotropin hypothesis proposes that excessive gonadotropin exposure is related to development of ovarian tumors (64). Many observations indicate that both normal human OSE cells, epithelial inclusions, and human benign and malignant ovarian tumor cells express receptors for FSH and LH/hCG (38, 72-77). FSH and LH/hCG have been reported to enhance cell proliferation of primary human OSE (76), primary ovarian cancer cells in culture (78, 79) and ovarian carcinoma cell lines (48, 80), however another study showed an anti-proliferative effect of FSH, and the absence of effect by LH on cell proliferation of OSE (81). These pieces of evidence suggest that high circulating levels of pituitary

gonadotropins may increase the risk of ovarian cancer by stimulating the growth of ovarian epithelial cells.

Sex steroid production is one of the major functions of ovarian cells. Most of the epidemiologic risk factors for EOC mentioned above and protective factors are related to the changes of sex steroid levels in women. The sex steroid hormonal hypothesis proposes that excess androgen stimulation of the OSE leads to increased risk of cancer, whereas progesterone stimulation of the OSE is protective of EOC (61). Steroid hormone receptors (*i.e.*, ER, PR, and AR) have been detected in human OSE (30, 31) and with varying levels of expression in ovarian tumors (28, 74, 82) and ovarian carcinoma cell lines (75, 83-85). Androgens are the main steroids produced by the postmenopausal ovary (86). Testosterone-stimulated growth of OSE cells in guinea pigs caused the formation of benign epithelial ovarian neoplasms (86). Progesterone can inhibit proliferation of some primary cultures of human OSE (2), although a similar study from another group found no effect on proliferation of progesterone (30). Still, the evidence that the progestin-only oral contraceptive pill, which does not suppress ovulation, decreases EOC risk to the same or greater degree than that seen with the combined contraceptive pill (87), and that progesterone can induce apoptosis in the OSE of monkeys *in vivo (88)*implicate a protective role of progesterone. The effects of estrogen on tumorigenesis assume complexity. Estrogens, taken as oral contraceptives during premenopausal years are protective but when used during postmenopausal years as hormone replacement therapy (89), estrogen may increase the risk of ovarian cancer (61, 90-93). Though human OSE cells in culture are reportedly unaffected by estradiol (2), continuous exposure to estradiol stimulates proliferation of sheep (94) and rabbit OSE cells and results in the formation of a papillary ovarian surface resembling human serous neoplasms of low malignant potential (95). Exogenous estrogen stimulated the growth of several ER-positive ovarian carcinoma cell lines in vitro (96-98). In contrast, other studies showed that exposure of some ovarian cancer cell lines to estradiol resulted in antiproliferative effects, including apoptosis and up-regulation of the tumor suppressor gene p53 (79, 99). However, the findings that estrogen reduces GnRH receptor expression in both OSE and ovarian cancer cells, thereby suppressing the growth inhibitory effects of GnRH (100) may indirectly indicate that estrogen increases ovarian cancer risk.

The inflammatory hypothesis is based on epidemiological studies, in which, many inflammatory factors have been indicated as ovarian cancer risk factor, such as exposure to asbestos and talc particles (101-103), pelvic inflammatory disease (104,

105), endometriosis which is the presence of endometrial tissue outside the endometrium, which causes a marked local inflammatory reaction (103, 106-109). This hypothesis is consistent with the known protective effects of tubal ligation and hysterectomy on ovarian cancer risk (59), because they disrupt the pathway by which the inflammatory exposures may reach the OSE cells (110). In fact, many inflammatory cytokines, growth factors, chemokines, as well as infiltrating macrophages and T cell have been found in ovarian tumors (111-114). This hypothesis is also supported by the studies, which demonstrate that the risk of EOC is reduced in women who are consistent users for at least 6 months of low dose aspirin, acetaminophen or non-steroidal anti-inflammatory agents (115, 116).

Normal human OSE cells produce TGF-β, which acts as an autocrine growth inhibitor (42, 117). The proliferation of various ovarian cancer cells has also been demonstrated to be inhibited by exogenous TGF-β, including primary ovarian cancer cells from solid tumors and patients' ascites (42, 43) as well as some ovarian carcinoma cell lines (42, 118). However, some ovarian cancer cells, despite appropriate TGF-β-induced Smad signaling (119), were resistant to the growth-inhibitory effects of TGF-β and /or did not produce TGF-β (42, 43), pointing to a mechanism for escape from the negative growth regulation by TGF-β during tumor progression. In addition, TGF-β has also been shown to induce apoptosis in ovarian cancer cells (117, 120), but not in OSE cells (117).

Though most of the ovarian cancers are caused by sporadic mutations, a strong family history of ovarian cancer is still an important and the best-defined risk factor, which is due to 5-10% ovarian cancer incidence. All these hereditary ovarian cancer are associated with germline mutations, primarily in BRCA1 and BRCA2 (121).

4. Tight Junction

General Introduction of TJ

An epithelium is characterized by its ability to form selective barriers between tissues and different body compartments and by its polarity. The tight junction (TJ) is a crucial structure of epithelium, since it mediates adhesion between epithelial cells, controls paracellular permeability across epithelial cell sheets (barrier function) (122) and restricts intramembrane diffusion of lipids in the plasma membrane (fence function) (106, 123) to maintain the epithelial polarity (*i.e.* to maintain an apical and a basolateral cell surface domain) (Figure 3). More recently, TJs have been shown to

harbor evolutionarily conserved protein complexes that regulate polarization and junction assembly (124) and to recruit signaling molecules that participate in the regulation of cell proliferation, differentiation, and gene expression (125).

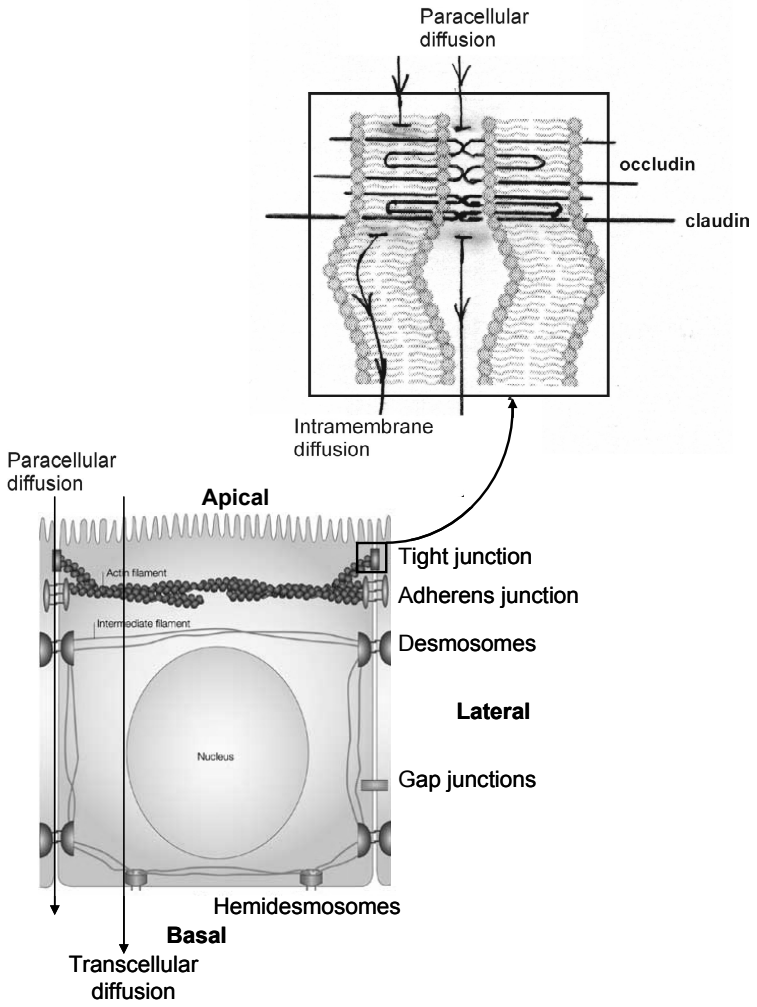

Figure 3. A schematic representation of a polarized epithelial cell. The different types of intercellular junctions, as well as hemidesmosomes are shown. Tight junctions and adherens junctions are linked to the actin cytoskeleton. Magnification shows the bilayer lipid membranes of two adjacent epithelia. Tight junction controls the paracellular and intramembrane diffusions of molecules and ions via TJ proteins i.e. occludin and claudin. (Adapted from Matter et. al., 2003) (125)

TJs consist of transmembrane proteins *i.e.* occludin, claudins, junctional adhesion molecules (JAMs), CRB3 and other single-span tansmembrane proteins; and peripheral membrane proteins, including ZO-1, ZO-2, ZO-3, MAGI proteins, cingulin, ZONAB and others (122). Transmembrane proteins of the TJs bind via their intracellular domains to peripheral membrane proteins, thereby allowing the transmembrane proteins to organize themselves in the membrane, to attach to the

cytoskeleton and to initiate cell signaling (Figure. 4). Some of these TJ proteins, which we have studied in this thesis, will be further introduced below.

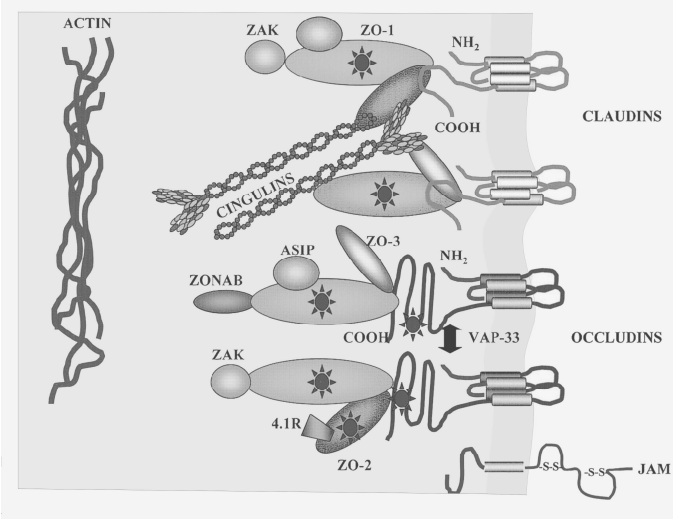

Figure 4. Proteins of the tight junction (TJ). Sun symbols indicate phosphorylation.(Modified from Cereijido et al., 2000) (126).

ZO-1

ZO-1 was the TJ component, which was first identified (127), and was subsequently found to be localized also in adherens junctions of cells that lack TJs (128, 129). ZO-1 has several protein-protein interaction domains: three PDZ domains, one SH3 domain, and one catalytically inactive guanylate kinase (GUK) homologue. The protein ZO-1 can interact with the transmembrane proteins JAMs, claudins, and occludin. Moreover, it forms stable complexes with either ZO-2 or ZO-3 via a PDZ-PDZ domain-mediated interaction, binds to other adaptors such as cingulin, and contains a discrete actin-binding domain in its C-terminal half (130).

Occludin

Occludin is the first transmembrane protein of TJ to be identified in chicken liver (107) and in mammalian species 8601611. TJ has been shown to be involved in cell adhesion (108), TJ barrier and fence function (109). Cloning and sequencing of the corresponding cDNAs revealed that occludin has four transmembrane domains, three cytoplasmic domains and two extracellular loops (131) (Figure 5).

Many studies have strengthened the concept that occludin is a true TJ component, such as that overexpression of occludin in cultured MDCK cells increased the number of TJ strands (132) and that either overexpression of occludin in insect Sf9 cells (133) or in mouse L fibroblasts (134), result in formation of short TJ-strand-like structures

in the cytoplasmic vesicular structures and at cell-cell borders, respectively. However, the findings that when occludin-deficient embryonic stem cells were differentiated into epithelial cells by formation of embryoid bodies, well-developed TJ structures were formed between adjacent epithelial cells lacking occludin 9548718, indicates that occludin is not required for the formation of TJ strands..

Nevertheless, occludin is not only a structural component of TJ; it also plays an important role in TJ function. Firstly, in transfected fibroblasts, occludin was reported to show cell adhesion activity (108). Secondly, expression of a dominant negative mutant of occludin leads to disturbance in the intramembrane fence that restricts diffusion of lipids between the apical and basolateral cell surface domains. Thus, occludin is also involved in the maintenance of cell surface lipid polarity. Thirdly, overexpression of occludin was shown to increase size-selective paracellular permeability and decrease ion conductance (132).

Recent studies have suggested that occludin has an important role in targeting TGFβ receptors to the TJ. This may be important for the TGF-β mediated epithelial-to-mesenchymal transition, which requires loss of polarity and dissolution of junctions (135, 136).

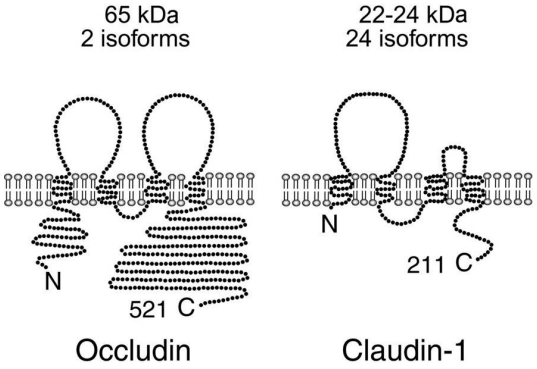

Figure 5. Integral membrane TJ proteins occludin and claudin-1. Both occludin and claudin-1 are tetraspan proteins that share no sequence homology. (Adapted from Schneeberger et al., 2003) (137).

Claudins

To date, 24 members of the claudin family have been identified in mouse and human, mainly through database searches (138, 139). These proteins, like occludin, also have four transmembrane domains, though they do not show any sequences similarity to occludin (Figure 5). The expression pattern of claudins varies considerably among tissues (138, 140). Some claudins, such as claudin-5 and claudin-11, have tissue-specific expression patterns (141, 142). Most cell types, however,

express more than two claudin species in various combinations to constitute TJ strands. Claudins interact with each other between different TJ strands or within individual strands in a homotypic as well as heterotypic manner (143). The C-terminal amino acids of claudins encode PDZ-binding motifs, and these motifs are highly conserved throughout the claudin family. Through these PDZ-binding motifs, claudins directly interact with peripheral PDZ-domain-containing proteins, including ZO-1, ZO-2, ZO-3 and other cytoplasmic TJ associated proteins (144) (Figure 4)

Claudins are the main proteins important for TJ strand formation. The most convincing evidence is that expression of a single claudin type is sufficient to induce the appearance of TJ-like intramembrane strands in fibroblasts, suggesting that they are important structural components of TJs (134). This is also supported by the disappearance of junctional intramembrane strands in central nervous system myelin and Sertoli cells in claudin-11 null mice (145).

Claudins also appear to be primarily responsible for the formation of ion-selective paracellular diffusion (which could be measured by trans-epithelial resistance) pathways: *in vitro,* experimental results showed *de novo* or over-expression of claudin-4, -7 and -8 induced higher resistance in cultured cell models (146-149). This is in accordance to the findings *in vivo,* that these claudins are located only in distal nephron segments with high resistance (150). Likewise, claudin-2 induces lower resistance (151) and is found *in vivo* in leaky epithelia, such as the proximal renal tubule (152) and intestinal crypts (153).

5. Tight Junction in Human Tumors

Altered TJs in Human Tumor

Numerous studies have shown that alterations in the number and appearance of TJs are associated with many human diseases, including various primary human tumor types. In this thesis, some alterations of TJs in human tumor cells compared to normal adjacent cells or the tissue wherein the tumor arose (Table 1) are listed. It can easily be seen that the alterations vary between different types of tumors.

Table 1. Tight junction proteins expression in human tumors

cancer type	ZO-1	claudin-1	claudin-2	claudin-3	claudin-4	claudin-7
Colorectal		↑ (154, 155)		↑ (154)	↑ (154)	
Breast	↓ (156)	↑ (157)		↑ (158)	↑ (157, 158)	↓ (159, 160)
Proststate					↑ (161)	
Pancreas		↑ (162)			↑ (163)	
Cervix		↑ (164)	↑ (164)		↑ (164)	↑ (164)
Ovarian					↑ (165, 166)	

↑ : up-regulated protein compared with the normal tissue.

↑ : down-regulated protein compared with the normal tissue.

The Role of TJ Alterations in Tumorigenesis and Tumor Progression

The role of TJ alternations in tumorigenesis and tumor progression is still far from being completely understood. Though, some studies implicate that changes in some TJ proteins might effect migration, polarization, invasiveness of normal cells and/or tumor cells (154-158). For example, Michl *et al* (157)have shown that, within two subclones cell lines, derived from the same primary pancreatic tumor, the one with high metastatic and invasive potential exhibited very weak claudin-4 expression, compared to the other one with low invasiveness. *In vitro* overexpression of claudin-4 in this highly invasive cell line leaded to significantly reduced invasiveness and inhibited colony formation in soft agar assays. Furthermore, tailvein-injected claudin-4 overexpressing cells in mouse formed significantly less pulmonary metastases in comparison with mock-transfected cells. However, no effects of TJ alternations on cell proliferation and apoptosis were detected in these studies.

6. Adherens Junction

General Introduction of AJ

The adherens Junction (AJ) is another member of intercellular junctions. The AJs form continuous adhesion belts localized near the apical end of the cell, just below TJs (Figure 3). The key transmembrane proteins of AJs belong to the cadherin family,

which is Ca^{2+}-dependent and consists of over 80 members (159). The catenins, cytoplasmic proteins of AJs form a complex with the intracellular portion of the cadherin molecule. Epitheilal (E)-cadherin and neuronal (N)-cadherin are two well characterized classical cadherins. The cytoplasmic tail of them is linked to the actin cytoskeleton and other signaling elements commonly through binding to catenins (*e.g.* β-catenin) (160). It was described that unbound β-catenin could enter into the cell nucleus, interact with transcription factors, and regulate gene transcription through a Wnt signaling pathway (161). Thus AJs is not only a structure to keep cell-cell adhesion, it could also play roles in intracellular signaling pathway via regulation of cadherin-catenin binding and further to control the number of free cytoplasmic β-catenin.

E-cadherin, which is primarily expressed in epithelia, has also been speculated to act as a precursor for the establishment of TJs. A recent study has shown that mice lacking E-cadherin die shortly after birth because of dehydration. A closer molecular examination of the skin biopsy of these E-cadherin deficient mice revealed that key TJs components are improperly localized, and impaired TJ function was shown via altered resistance in the granular layer. Besides, an earlier *in vitro* study has also shown that E-cadherin is crucial for the assembly of TJs (162).

E -cadherin and N -cadherin in OSE and EOT

E-cadherin, which is commonly expressed in epithelia, is constitutively present in human oviductal, endometrial and endocervical epithelia (163) and also in mouse and porcine OSE (164, 165). In contrast, E-cadherin expression in human OSE is limited to the rare regions where the cells assume columnar shape, cleft formations and inclusion cysts, *i.e.* where they approach the phenotype of metaplastic epithelium (5, 6, 17). E-cadherin was also detected more frequently in cultured OSE from patients with a family history of ovarian cancer compared to OSE from control patients (22). Moreover, expression of E-cadherin was also found in benign adenomas, borderline tumors, well-, moderately- and poorly-differentiated adenocarcinomas of the ovary (6, 166-170). However, in one of these studies, E-cadherin was not found in all poorly-differentiated adenocarcinomas samples included in that study (170).

In the human, OSE, granulosa cells, and extraovarian mesothelium are connected by N-cadherin, which characterizes adhesive mechanisms of mesodermally derived tissues (5, 22, 171, 172). Peralta Soler *et al.* showed that N-cadherin was co-expressed with E-cadherin in serous and endometrioid ovarian adenocarcinomas (168).

7. Modulation of TJs and AJs by TGF-β1

Transforming growth factor (TGF) –β1 is a polypeptide member of the Transforming growth factor beta superfamily of ligands. TGF-β signals through two transmembrane serine-threonine kinases, the type II (TβRII) and type I (TβRI) receptors. In addition to its growth inhibitory function (173), TGF-β1 has also been demonstrated as one of the main factors to induce EMT accompanied with the loss of E-cadherin (174-178). Induction of EMT, including the repression of TJ proteins during TGF-β1 stimulation was found in pig thyrocytes (179) and claudin-4 was negatively regulated by TGF-β in pancreatic cancer cells through inhibition of the Ras signalling pathway (157). The classically described TGF-β pathway begins with the binding of the TGF-β ligand to the constitutively active TβRII, which in turn binds and phosphorylates TβRI. This activates the Smad pathway to regulate gene transcription. It has been recently indicated that TGF-β-induced cell cycle arrest and migration, but not EMT are abolished after silencing of Smad4 and TGF-β-dependent EMT is required both Smad-dependent and Smad-independent pathways (180). This evidence suggests that the growth inhibitory function and EMT can be induced by TGF-β via different cell signaling pathway.

AIMS OF THIS STUDY

The tumorigenesis and tumor progression of epithelial ovarian tumors is largely unknown. This leads to limitations in i.e. early clinical diagnosis and efficient treatment. Tight junctions have been studied for a long time since they are important to keep the barrier function and polarization of normal epithelium. A growing body of evidence has also implicated that tight junction might play roles in intra-cellular signaling pathways that regulate cell proliferation, polarization, and differentiation. Altered expression of different tight junction proteins have been discovered in many types of human epithelial derived tumor cells.

The overall aim of this study was to investigate the expression, localization, function and modulation of tight junctions in normal ovarian surface epithelium and epithelial ovarian tumors to further understand the possible roles of tight junctions in transformation of ovarian surface epithelium towards epithelial ovarian tumors.

The specific goals for the studies described in this thesis were:

- To study the expression pattern and localization of tight junction proteins, and the function of tight junctions in ovarian surface epithelium ***(paper I).***

- To study the expression pattern and localization of tight junction proteins in epithelial ovarian tumors in comparison to normal ovarian surface epithelium and inclusion cysts ***(paper II).***

- To study the expression pattern and localization of tight junction proteins, and the function of tight junctions in human ovarian cancer cell-lines derived from ovarian cancers of various histological-subtypes ***(paper III).***

- To study whether transforming growth factor-β1 can modulate the formation and function of tight junctions in ovarian surface epithelium and ovarian cancer cells ***(paper IV).***

METHODOLOGICAL CONSIDERATIONS

The methodology used in this thesis is outlined in the following schematic drawing and described in detail in the respective papers. In this section, considerations of some specific parts of materials and methods are commented.

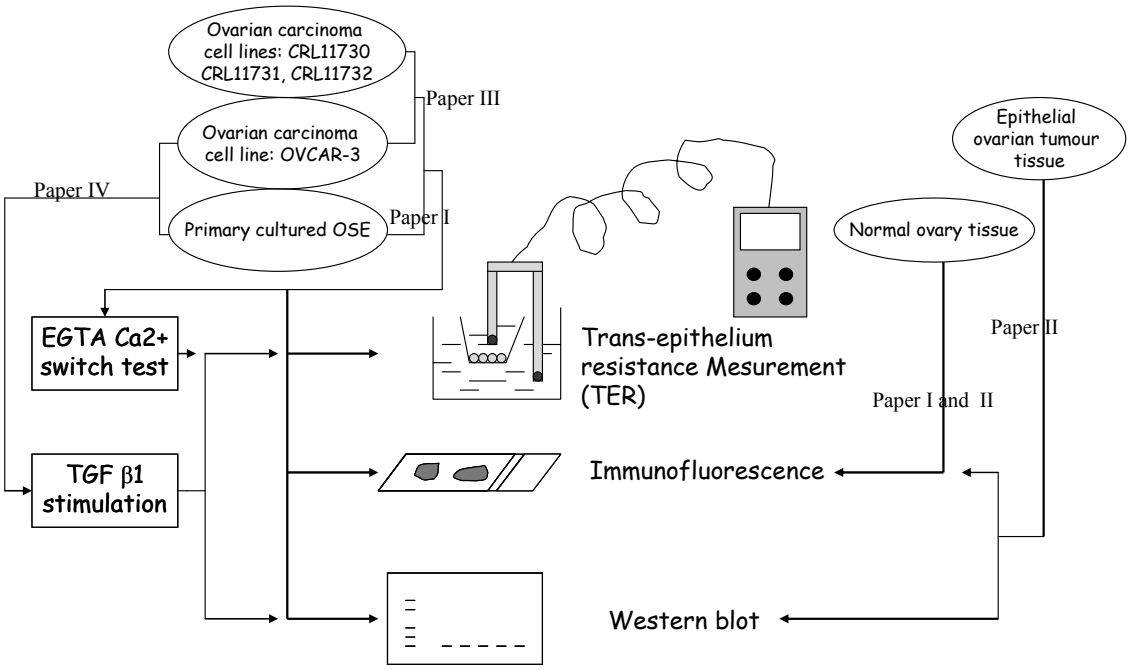

Figure 6. Schematic outline of materials and methods used in papers I-IV.

1. Patient Materials

The studies of the present thesis were approved by the Ethics Committee of the Sahlgrenska Academy at Göteborg University. Every participating woman was given both written and verbal information about the study. Informed written consent was obtained from all women before they were included.

Normal ovarian tissue biopsies (paper I and paper II) were obtained from thirteen (four pre-menopausal and nine post-menopausal) women operated on for non-ovarian diseases. Ovarian surface epithelia (OSE) were obtained from eighteen women.

2. Ovarian Surface Epithelium Cell Culture

Earlier passages of OSE cultured after intra-peritoneal brushings of the ovary are the main sources to observe biological characteristics of OSE. A gentle brushing of the ovarian surface efficiently reduces the risk of major injury and also makes the procedure simplified. It reduces the contamination of ovarian stroma cells. This method provides enough yields of cells for further experiments and can mimic physical stage of cells, *e.g.* cell-cell attachment.

As a drawback in all *in vitro* culture systems, the cells are exposed to un-physiological culture conditions. This means that culturing cells leads to the risk that it could not exactly represent *in vivo* situation. In the culture system used in the present study, diversity of cell phenotype was seen: instead of the typical cobblestone morphology, some cultured cells acquired fibroblast-like appearance. This was found both in the first passage and the later passages of the culture. The same phenomenon was also described in studies from other groups (9, 12, 13). The reason might be either contamination of stroma or epithelio-mesenchymal transition (EMT) of OSE. We compared the histological characteristics of fibroblast-like cells and OSE with cobblestone morphology *in vitro.* The former cells differed greatly from OSE *in situ, i.e.* they weakly expressed cytokeratin 8, ZO-1 and occludin. Moreover, the staining of ZO-1 and occludin were scattered in the cytoplasm instead of restricted to the cell border (Figure 7) (our unpublished data). For this reason, these cultures with cells of fibroblast-like appearance were not used.

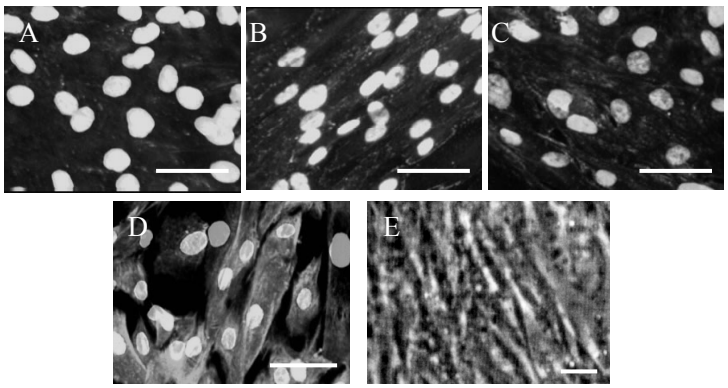

Figure 7. Phase-contrast microscopic analysis of cultured OSE with fibroblast-like appearance (E), immunofluorescence microscopic analysis of the same fibroblast-like OSE stained with cytokeratin 8 (A), ZO-1 (B), occludin (C)and vimentin (D). Bar=50μm

3. Trans-epithelial Resistance Measurement

One important role the tight junction (TJ) plays is its barrier function, which restricts the ions and hydrophilic nonionic molecules to diffuse along the paracellular pathway in a manner that depends on the charge and the size (181-183). Trans-epithelial resistance (TER) measurement (used in paper I, III and IV) is often performed to detect the ion permeability of TJ (184). Briefly, the cells were grown on Transwell filter inserts with a membrane pore size of 0.4μm. Subsequently TER was measured every 2-3 days by the Millicell Electrical Resistance System (ERS) electrodes and meter (shown in figure 6) according to the manufacture's instruction.

In fact, TER is a composite of transcellular and paracellular (Figure 3) resistance. However, in most low-resistance epithelia (less than 1000Ω), the electrical resistance of the paracellular route is much lower than the transcellular resistance (185-187). Since the two pathways are arranged in parallel, $1/TER = (1/R_{transcellular}) + (1/R_{paracellular})$; the measured TER essentially reflects paracellular resistance, but not transcellular resistance in our cell models.

Here we should also consider that the paracellular resistance is the sum of the junctional resistance and the resistance along the paracellular space, which means that a collapse of the paracellular space can cause a non-ion-selective increase in TER. However, our TER data on fibroblast-like cells in comparison to OSE cells and ovarian cancer cell lines, showed that TER in fibroblast-like cells never reached to 15 $\Omega*cm^2$ within 27 culture days, even though these cells became confluent at day 15. In OSE cells, as described in Paper I, TER maximum value varied in the range of 30-70 $\Omega*cm^2$ within 15-27 culture days (cells acquired confluence in about 12 days). In OVCAR-3 cancer cell line, TER value already increased beyond 100 $\Omega*cm^2$ when cells only reached half confluence (unpublished). As described above, fibroblast-like cells do not have proper TJ formation, OSE cells only expresses ZO-1 occludin and claudin-1 (Paper I, II), but OVCAR-3 cells expresses two more TJ proteins, claudin-3 and claudin-4 (paper II). This data suggests that in our studied cell model, the paracellular space has minimal effect on TER compared with TJ formation.

However, there may still be disadvantages with this detection system. Firstly, the disturbance of cultures by change of medium, changes in pH value, temperature and the volume of medium could result in large fluctuations in TER (188). Therefore, when we change culture medium, it is necessary to gently aspirate the old medium without touching the cell layer and gently blow out the fresh culture medium along

with the lateral wall of inserts. We commonly added 250µl medium to the inner insert chamber and 950µl to the outer chamber to reach the same level of medium in each chamber. It is also known that the cultures need to be equilibrated for at least 15min at room temperature prior to the measurement. Secondly, the electrodes we used could result in cross-contamination of the cultures. To avoid this, the electrodes were immersed in 70% ethanol for 15 min before the measurement, and this was followed by equilibration in culture medium for 5min. Before the measurement was switched to another group of cells, the electrodes were immersed in ethanol for 5min and equilibrated in culture medium. By carefully performing all these steps, the development of TER in cell cultures over days could be followed and reproducible values can be achieved.

4. Immunofluorescence

Indirect immunofluorescence has been widely used to investigate the assembly and localization of TJ proteins (189). Comparing with DAB staining, immunofluorescence makes the membrane staining easier to be visualized and makes it feasible to investigate several transmembrane TJ proteins simultaneously. Though, the photobleaching is always a significant problem with immunofluorescence. Moreover, when immunofluorescence is performed on tissue sections, it is necessary to have the comparable H&E staining section, which will provide histological details

From our experience, frozen tissues sectioned in a cryostat are more suitable for immunofluorescence than paraffin-embedded tissues, since we consistently achieve stronger and more specific signals from the former. It is probably due to the excellent antigen preservation in frozen tissue sections. However, one disadvantage of frozen tissue sections is that they give less morphological details and resolution.

A good fixation is necessary to get good results of immunofluorescence. This means immobilization of antigens, while retaining authentic cellular and subcellular architecture and permitting unhindered access of antibodies to all cells and subcellular compartments. In the present study, cold acetone was used as fixative. This fixative dehydrates the cells, precipitates the protein molecules on the cellular architecture and therefore makes a good adhesion of them to the slides. The other advantage of acetone fixation compared with cross-linking reagents is that it keeps higher antigenicity of cell components and has a capability to permeabilize the membrane without dissolving phospholipids (190). The drawback of acetone fixation is less preserved

cell structure. We found the shrinkage of cells occurred more frequently in cytokeratin 8 (CK8) structures (*i.e.* we often found big gaps without staining between two CK8 positive stained neighboring cells) than in trans- membrane junctional structures (*i.e.* we usually found only one cell border stained with antibody between every two adjacent cells) (see Figure 2B and Figure 2D in paper I). It is worth to mention that fresh acetone must be used for fixation, as acetone will absorb more water if it is repeatedly used, and traces of water in the acetone ruin the tissue morphology and lead to higher backgrounds.

It is advisable to run the appropriate negative controls. Negative controls establish background fluorescence and non-specific staining of the primary and secondary antibodies. We ran the negative control with absence of primary antibody to discover the possible background from the non-specific staining of the secondary antibody. In the tissue section staining, the negative staining of stroma cells can exclude the possibility of non-specific primary antibody staining.

5. Western Blotting and Densitometric Scanning

The most essential issue of this semi-quantitative method used in the present study is the yield of proteins extracted from the tissue samples (paper II). The simple endogenous loading control marker like β-actin could not represent the exact amount of tumor epithelial protein from the tissue, since the tissue consists of tumor epithelia, tumor stroma and endothelia as well. Thus, when we compare the expression levels between different tissues, the results should be interpreted carefully. In an attempt to solve this problem, we used antibody towards cytokeratin 8, which has been proved to be expressed only in epithelia, but not in stromal cells and other type of cells within ovary tissues (Figure 8) (unpublished data). However, to my knowledge, there is no published paper describing whether CK8 is equally expressed in all types of epithelial ovarian tumor cells. Thus an optimal endogenous protein marker for both normal OSE and epithelial tumor cells was not found by us.

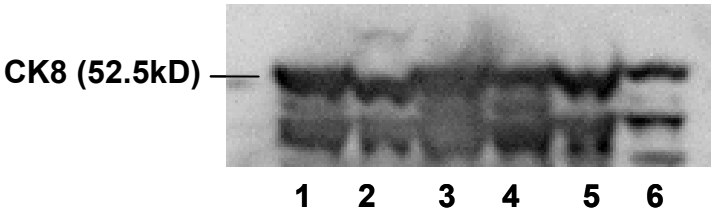

Figure 8. A representative immunoblot of epithelial ovarian tumors: benign adenomas (1, 2), borderline-type tumors (3, 4) and malignant adenocarcinomas (5, 6),

was probed by monoclonal anti-cytokeratin 8. Proteins were extracted directly from human tumor tissues and each protein lysate sample was loaded equally.

In Paper II, we used a large number of tumor samples, and it was not possible to analyze all of them on one gel. To normalize the samples data from different blots, we loaded one reference sample with the same volume on each gel, and we normalized all samples data to the reference before the statistical analysis.

6. TGF -β1 Treatment

This study was focused on whether transforming growth factor (TGF)-β1 can modulate TJ proteins and function. Based on previous studies showing that TGF-β1 can affect cell barrier function at doses of 1-100ng/ml (191, 192), we selected three doses (1ng/ml, 10ng/ml and 100ng/ml) of TGF-β1 on OVCAR-3 cancer cell line in our pilot study. A significant change of trans-epithelial resistance (TER) value was only found in the cells under the treatment at the dose of 100ng/ml (data not shown). Therefore, we final choose the 100ng/ml dose of TGF-β1 in our study.

To our experience, when cultured OSE cells and OVCAR-3 got microscopically confluent, the TER value usually reaches to a middle or slightly higher level. It will increase more during several days after that. This means most TJ structure and function has already been built up when cells got confluent, but they still have some capacity to build up more TJs after confluence. With this consideration, TGF-β1 was added just when the cells got microscopically confluent. To facilitate the investigation of the changes, this may result from the regulation either to degradation or to assembly of TJ proteins by TGF-β1.

At day five, OSE acquired the most significant morphological change. Therefore, in our present study, we chose day five as a time point to analyze TJ proteins. Because of the limitation of OSE samples, we were not able to choose more time points. Some optimizations like collecting proteins from the cells cultured in small trans-well inserts might be a good approach to analyze proteins from several time points.

SUMMARY OF THE RESUTLS

1. Formation and Barrier Function of Tight Junctions in Human Ovarian Surface Epithelium (Paper I)

The results of this study show, that the TJ proteins ZO-1, occludin and claudin-1 are localized to the OSE cell borders both in ovarian biopsies and in cultured OSE. The TJ structure was also visualized in early cultures of OSE via electron microscopy observation. This TJ was found to have weak ion-barrier function represented by low trans-epithelial resistance (TER), which could be interfered with by the Ca^{2+} chelator EGTA.

2. Differences in Expression Patterns of the Tight Junction Proteins, Claudin-1, -3, -4 and -5, in Human Ovarian Surface Epithelium as Compared to Epithelia in Inclusion Cysts and Epithelial Ovarian Tumors (Paper II)

The results of this paper show that claudin-3 and -4 are expressed *de novo* or up-regulated in ovarian epithelial inclusion cysts and ovarian serous and mucinous tumors as compared with normal OSE. Moreover, in ovarian serous and mucinous tumors, claudin-4 is significantly up-regulated in borderline-type tumors and malignant tumor compared with benign tumors; claudin-3 is significantly up-regulated in malignant tumors compared with benign and borderline-type tumors whereas no changes are found for expression of claudin-1 or -5.

3. Tight Junction Formation and Function in Serous Epithelial Ovarian Adenocarcinoma
(Paper III)

In this study, it was found that ZO-1, claudin-1, -3, -4 and E-cadherin are expressed along the entire cell periphery in ovarian serous adenocarcinoma cells, which is paralleled by a high TER value. Clear-cell and endometrioid ovarian adenocarcinoma cell lines with minimal TER value do not express claudin-4, and express only low levels or no E-cadherin.

4. TGF-β1 Modulates Tight Junction and the Expression of Cadherins in Cultured Ovarian Surface Epithelium and Epithelial Ovarian Cancer Cells (Paper IV)

The results of this study showed for the first time that TGF-β1 induced morphological changes in cultured OSE, which resembles an epithelial-mesenchymal transition (EMT) concomitant with an up-regulation of N-cadherin, down-regulations of claudin-1 and occludin. In OVCAR-3 cells, claudin-3, -4, and E-cadherin were down-regulated by TGF-β1, while claudin-1 was up-regulated, though morphological changes were not noted. Moreover, a decrease of TER in both TGF-β1 treated OSE and OVCAR-3 cells was found.

DISCUSSION

Tight Junction in Ovarian Surface Epithelium

For a long time, the knowledge of tight junction (TJ) in ovarian surface epithelium (OSE) was only limited to the ultra structural description arising from studies utilizing electron microscopy observation (193). These studies indicated that the presence of terminal TJs was seen on the adjacent lateral borders near the free surface of human OSE *in situ*. In paper I, TJ proteins and the ion-barrier function of TJ in OSE were studied. It was found that the TJ proteins ZO-1, occludin and claudin-1 were expressed on the border of normal OSE *in situ* and *in vitro*. The morphological structures were also confirmed by our electron microscopic observation. Moreover, the finding that cultured OSE in early passages built up low trans-epithelial resistance (TER), which could be interfered with by the Ca^{2+} chelator EGTA, represents a weak ion-barrier function of TJ. The expression and typical localization of TJ proteins confer a defining characteristic of epithelia to OSE. However, the low TER value compared with primary thyroid epithelial cells (194) implicates that the well-built barrier function of an OSE monolayer is not necessary for the physiological circumstance. Rather, the weak barrier function is more likely to be concomitant with weak adherence between cells, which in turn renders the cell layer susceptible to rupture and breakdown during ovulation.

Tight Junction in Ovarian Tumorigenesis

Recently, the profile of gene expression of ovarian carcinoma and normal OSE from SAGE study identified that genes encoding TJ proteins claudin-3 and claudin-4 to be two of the most highly up-regulated genes in serous epithelial ovarian cancer (195). Later on, studies using a variety of approaches such as microarrays, tissue arrays, and reverse transcription-PCR; and our study (paper II) using the immunofluorescence and densitometric analysis of western blots confirmed the up-regulation of claudin-3 and -4 in epithelial ovarian tumor (EOT) (196-201) at both mRNA level and protein level. We and another group (198, 202) also found that claudin-3 and -4 were expressed *de novo* in ovarian epithelial inclusion cysts compared with normal OSE. Furthermore, claudin-3 and claudin-4 were found to be increased with the increasing grade of epithelial ovarian tumors. Concerning claudin-1 or -5 expressions, no differences were detected within all the tumor samples.

These observations lead to the question whether changes in claudin expression are causes and / or consequences of tumorigenesis and tumor progression. Some clinical observational studies show that changes of claudin expression are related to increased invasion and poor survival in some types of tumors. For examples, in a tissue microarray study of gastric adenocarcinomas, an up-regulation of claudin-4 was found to be associated with decreased survival (203). In human pancreatic cancer, it has been reported that claudin-4 is expressed at significantly higher frequency in invasive cancers than in noninvasive pancreatic cancers (204).

It was shown that inhibition of claudin-1 leads to a increase of anoikis (a form of apoptosis, which is induced by detaching of the cells from the surrounding extracellular matrix); and a decreased anchorage independence, which could also be increased by claudin-1 over-expression in human colon cancer cell lines *in vitro*. Moreover, claudin-1 expression has significant effects on promoting growth of xenografted tumors *in vivo* (205). In immortalized human OSE cells, over-expression of claudin-3 and 4 results in increased cell survival, migration and mobility (206). However, expression of claudin-4 in human pancreatic cancer cells suppressed anchorage-independent growth (157). Similarly, in human gastric cancer cell lines, over-expression of claudin-4 induced by nonsteroidal anti-inflammatory drugs leads to suppression of anchorage-independent growth and cell migration (158). Though, manipulation on the expression levels of claudin-1or/and claudin-3 and -4 in all above mentioned studies did not lead to changes of cell proliferation *in vitro*.

It has also been demonstrated that claudins are able to interact with membrane-type matrix metalloproteinases (MT1-MMP), to promote MMP activity, and are also capable to change the cell invasiveness within different types of cell lines. A study on human embryonic kidney cell demonstrated that claudins could recruit all MT1-MMP and pro-MMP-2 to the cell surface to achieve elevated focal concentrations. Expression of claudin-1, 2, 3 and 5 were also proved to activate pro-MMP-2 in these cells (156). A study concerning claudin-1 in human colon cancer showed that claudin-1 played a causal role in increasing MMPs activity *in vitro* and tumor invasiveness both *in vitro* and *in vivo* (205). Similarly, in a recent study of oral squamous cell carcinoma cells, a stronger expression of claudin-1 together with higher activation of MMP-2 and MMP-9 were found in highly invasive cell lines compared with weakly invasive cell line; moreover, claudin-1 siRNA decreased the activation of MMP-2 and the expression of MMP-2 activator MT1-MMP, and suppressed the cell invasion. On the other hand, claudin-4 was strongly expressed in weakly invasive cells, and

knockdown of claudin-4 by siRNA increased cell invasiveness (207). In another study, elevated levels of the active form of MMP-2 together with increased cell invasiveness and migration were found when immortalized normal human ovarian surface epithelial cells were transfected with claudin-3 or claudin-4. In the same study, siRNA knockdown of claudin-3 and 4 in ovarian cancer cells reduced cell invasiveness, though it did not lead to a decrease of MMP-2 activity (206). However, the study of human pancreatic cancer cells (157) demonstrated that claudin-4 was strongly expressed in the cells with low metastatic and invasive potential, whereas it was weakly expressed in the highly invasive cells. Moreover, over-expression of claudin-4 in pancreatic cancer cells reduced cell invasiveness *in vitro* and formed significantly less pulmonary metastases in mice compared with control group *in vivo*, although over-expression of claudin-4 did not lead to changes in MMP-2 activity.

Thus, it is clear that claudin expressions are linked to tumor invasion and metastasis. Especially, claudin-3 and -4 are likely to enhance the migration and invasiveness of ovarian cancer cells, indicating that they might also play a role to promote ovarian carcinogenesis.

However, the above mentioned results also implicate a complexity in the correlation between claudins and, cell invasiveness and migration. The effects on MMP-2 activity and cell invasiveness of different claudins were diverse even within the same type of cells (207). Moreover, even the same claudin, such as claudin-4, also played the different roles with respect to cell invasiveness and migration in different type of cells (157, 158, 206, 207).This could be explained by tissue specificity and / or claudin type specificity. Alternatively, it could also be the results of different cell models and culture systems, which could differ from physiological circumstances. It should be emphasized that all these studies were performed on cell lines and in some of these studies, no *in vivo* data was provided. In any event, more comprehensive studies are still deserved. Whether the changes of invasiveness directly induced by alteration of claudins were mediated or only mediated via regulating activity of MMPs is also yet to be well defined.

Additionally, the molecular mechanisms involved in the events that claudins are relevant to cell survival, motility and invasion have only been studied at an early stage. The recent study mentioned above (205), has indicated that over-expression of claudin-1 in colon cancer cells lead to TCF-LEF/β-catenin activation, which is known to act as a transcriptional factor, inducing expression of important oncogenes, related to cell proliferation, survival and invasion (myc, cyclin D1, MMP-7). In the ovary, it

has been demonstrated that β-catenin is significant increased in human ovarian cancer compared to the normal ovary. Moreover, β-catenin and Lef-1 can be coimmunoprecipitated in ovarian tumours, but not in the normal ovary (208). We have already showed that both OSE and ovarian cancer cells express claudin-1, and that claudin-3 and -4 are up-regulated in ovarian cancer cells, whether these types of claudins could regulate the same and/ or other cell signaling pathway in OSE and ovarian cancer cells is an intriguing area of future investigation.

Compared with claudins, much more evidence has been shown that other proteins belonging to TJ family, *e.g.*, occludin, could participate in the signaling pathways that regulate epithelial differentiation and proliferation, thus, providing other indirect proofs that TJs play roles in tumorigensis. In a large proportion of cancers, loss of epithelial differentiation correlates with deregulation of Ras signaling. Over-expression of the junctional membrane protein occludin is able to suppress transformation of salivary epithelial cells induced by Raf-1, a common Ras effector (155). The finding that deletion of the occludin gene in mice affects the differentiation of some epithelial cell types (209) also suggests a role of occludin in cell differentiation. Though there is no direct evidence showing that occludin could regulate differentiation of OSE and epithelial ovarian cancer, our result that decrease of occludin expression was found in epithelial-mesenchymal transformation (EMT) induced by transforming growth factor (TGF)-β1 in OSE (paper III) implicates a potential role of occludin in cell differentiation of OSE.

Modulation of Tight Junction by TGF-β1 in Ovarian Surface Epithelium and Epithelial Ovarian Cancer cells

Up-regulation of TJ proteins claudin-3 and -4 has been frequently demonstrated in epithelial ovarian tumors (196-201, 210). The modulatory mechanism of TJ proteins in OSE and epithelial ovarian cancer (EOC) cells turns to be another important issue. In studies of Madin-Darby canine kidney (MDCK) cells (179), rat Sertoli cells (211) and human pancreatic cancer cells (157), TGF-β1 has been shown to regulate the expression of TJ proteins either synergistically with epidermal growth factor (EGF) or independently. In the ovary, TGF-β1 and its receptors TβRI and TβRII have been found in both OSE and epithelial ovarian cancer (EOC) cells (212). Though, the most extensive studies have been focused on direct growth inhibitory effects of TGF-β1 on OSE and EOC cells *in vitro*, only little is known in the aspect of other TGF-β1 effects, for examples, the modulation of TJ in OSE and EOC. Our data

in paper IV, demonstrated that expression of TJ proteins and ion-selective barrier function could be modulated by TGF-β1 treatment of human OSE and EOC cell line.

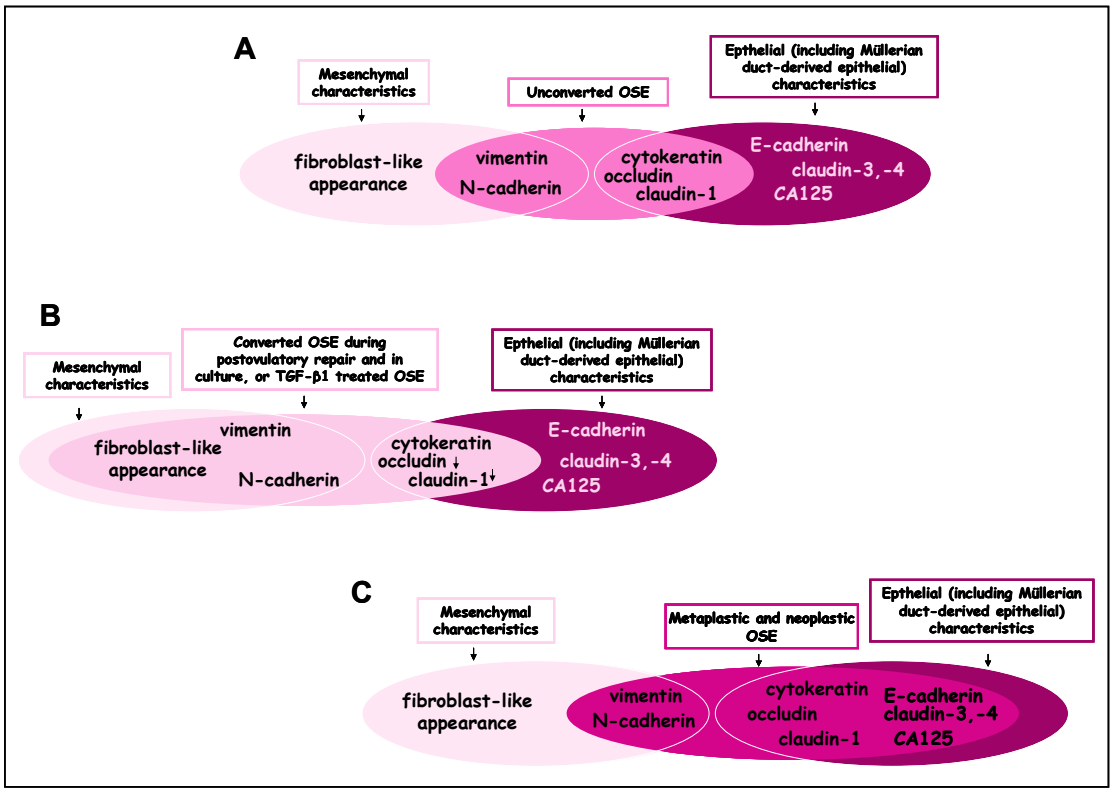

Figure 9. Different phenotypes acquired in OSE under various physiological, pathological and circumstances.

We found that expression of claudin-3 and -4 in OVCAR-3 ovarian cancer cell line, was down-regulated by TGF-β1 *in vitro*, implicating changes with the tendency towards normal OSE. These results are in line with the above mentioned investigation, that claudin-4 expression was down-regulated by either exogenously added TGF-β1 or constitutively over-expressed TGF-β1 in human pancreatic cancer cell line (157). Therefore, we speculate that TGF-β1 might function as an inhibitory regulator of claudin-3 and -4 in OSE. This function might be disrupted somehow during neoplastic progression. In turn, this disruption might account for the observed up-regulated claudin-3 and -4 in ovarian cancer. Our speculation is also supported by a recent investigation of genes expression profiles of ovarian cancer and OSE, showing that genes that inhibit TGF-β signaling are up-regulated in ovarian cancer, while genes that enhance TGF-β signaling are down-regulated compared with normal OSE samples (213).

In paper IV, the development of a spindle-shaped, fibroblast-like morphology was observed in TGF-β1 treated OSE, together with an increased expression of N-

cadherin, decreased expression of occludin and claudin-1 (Figure 9B). These changes have been early described as hallmarks of epithelial-mesenchymal transition (EMT) (174, 179, 214). In line with this we conclude that TGF-β1 can induce EMT in human OSE *in vitro*. As described in the introduction part of this thesis, OSE keeps a mixed epithelio-mesenchymal phenotype (Figure 9A) and multi-differentiation potential, which includes transition to mesenchymal phenotypes likely as EMT under physiologic conditions (*e.g.* wound healing process after ovulation)(Figure 9B); and the conversion from normal OSE to the aberrant epithelial cells that acquire more characteristics of the Müllerian duct-derived epithelia during metaplasia and neoplastic progression (1)(Figure 9C). The Müllerian-like differentiation of OSE is more likely as a continuation of OSE development. Our data suggest that TGF-β1 might be a potential regulator of OSE differentiation *in vivo*. TGF-β1 is present in the human follicle, and the expression intensity appears to increase in theca and granulosa cell layers as the follicle matures. Furthermore, in human luteal tissue, strong expression of TGF-β1 was found during early and midluteal phases, and reduced during late luteal phase (215, 216). This data indicates that TGF-β1 is accumulated at ovulation site in ovary during the period around ovulation, providing the possibility for TGF-β1 to induce EMT of OSE, which might become trapped within the ovary at ovulation. Conversely, it is conceivable that when TGF-β signaling is disrupted in ovarian cancer (213), then trapped OSE will keep its epithelial phenotype and predispose to aggregation and subsequent inclusion cyst formation. Our data on ovarian cancer cell line OVCAR-3 has shown that E-cadhiern was down-regulated by treatment of TGF-β1, whereas no EMT like morphological change was found. It might be due to the possibility that OVCAR-3 acquired a certain capability to incompletely escape from the negative regulation by TGF-β1. Collectively, we suggest that TGF-β1 might be a regulator responsible for preventing OSE to acquire some phenotypes, *i.e.* up-regulated expression of claudin-3, -4 and E-cadherin, which often appear in metaplastic and neoplastic OSE. And during tumorigenesis its negative regulation might be somehow inhibited by changes in its pathway. However, TGF-β1 has been introduced to play dual roles in ovarian tumorigenesis *in vitro*, since it has both growth inhibitory effects (42, 43, 117, 118) and the ability to enhance the cell invasiveness (217). This complexity becomes one of the main reasons counting for the fact that the real effects of TGF-β1 on OSE and ovarian cancer under physiological and pathological conditional are far from completely understood. Nevertheless, from another point of view, our study provides an approach to

investigate the downstream pathway of TGF-β1 induced TJ variations, which might be shared with other TJ modulators.

Clinical Significance of Tight Junction

It is interesting that claudin-3 and claudin-4 have been shown to represent the natural receptors for *Clostridium perfringens enterotoxin* (CPE) and to be the only family members of the transmembrane tissue-specific claudin proteins capable of mediating CPE binding and cytolysis (218, 219). CPE is a single polypeptide with a molecular mass of 35 kDa that causes food poisoning. CPE triggers lysis of epithelial cells through interaction with claudin-3 and -4, resulting in the initiation of massive permeability changes, osmotic cell ballooning and lysis (218-220). Recent study has shown that CPE can eliminate human ovarian cells *in vitro* in isolated primary cultures as well as *in vivo* when grown in the peritoneal cavity of mice. In this latter case, even chemotherapy-resistant primary cancer cells were susceptible to CPE-mediated cytolysis, and no adverse effects were observed throughout the CPE treatment (221). Taken together, these studies highlight the possibility to use claudin-3 and -4 as therapeutic targets in the treatment of human epithelial ovarian cancer.

CONCLUSIONS

1. Normal ovarian surface epithelia form tight junction with weak ion-barrier function represented by trans-epithelial resistance.

2. Tight junction proteins claudin-3 and claudin-4 are *de novo* expressed or up-regulated in ovarian epithelial inclusion cysts and ovarian serous and mucinous tumors with comparison to normal ovarian surface epithelium, and the expression levels of claudin-3 and claudin-4 increase with the malignancy of the epithelial ovarian tumors.

3. In four ovarian cancer cell lines, serous adenocarcinomas but not clear-cell or endometrioid adenocarcinoma have intact and well-functioning tight junctions and express E-cadherin.

4. Transforming growth factor -β1 can modulate the formation of tight junction and adherens junction, and the ion-barrier function of tight junction in normal ovarian surface epithelia and ovarian cancer cells *in vitro*. Moreover, transforming growth factor -β1 can induce epithelial-mesenchymal transition of cultured normal ovarian surface epithelia.

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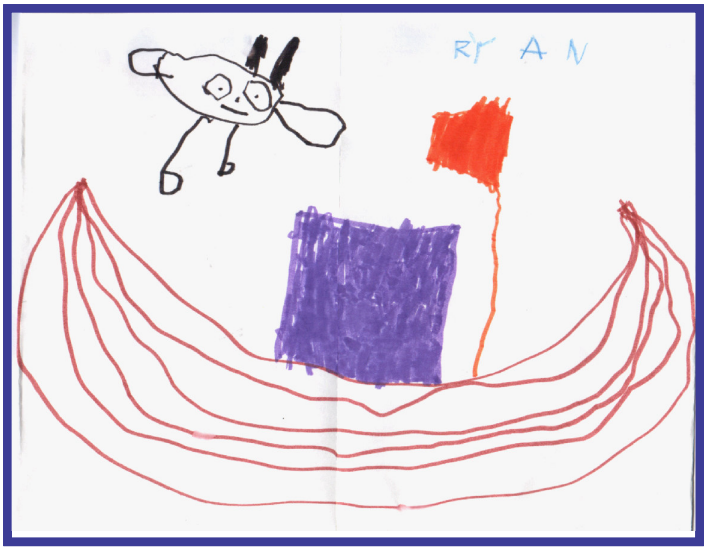
Wings of thinking --from Richard



The viking boat loaded with much braveness --from Ryan

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