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Differential expression of claudins in human endometrium and endometriosis

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Abstract
Membrane proteins of the claudin superfamily are important components of cellular tight and adherens junctions. Although their exact function remains unclear, these proteins may play a role in tissue remodeling, a process which is associated with several diseases including endometriosis. In the present work we analyzed the expression of 13 members of the claudin family in the endometrium and peritoneum by microarray analysis. Real-time polymerase chain reaction and immunohistochemistry in human endometrium and peritoneal endometriotic lesions were performed for validation of the expression of claudin-1, -3, -4, -5 and -7. Diminished expression of claudin-3, -4 and -7 in ectopic endometrium was frequently observed as indicated by all three methods. In contrast to a higher expression of claudin-5 mRNA detected in bulk biopsies of ectopic endometrium, immunohistochemistry revealed no alteration of claudin-5 protein expression in glandular cells of endometriosis samples. The downregulation of various members of the claudin family may contribute to endometrial cell detachment and increase the number of cells invading pelvic organs.

Keywords: *Endometrium, claudins, microarray*

Introduction

Claudins are multi-spanning membrane proteins forming the backbone of tight junctions which play an important role in both epithelial cell polarity and tissue permeability. The claudin family consists of more than 20 transmembrane proteins forming homomeric and heteromeric configurations. The expression pattern of claudins shows a distinct tissue distribution which may be used for histological differentiation of malignant tumors [1–3]. Modulation of tight junction structure has also been shown in tumorigenesis and invasiveness. Analysis revealed both up- and downregulation of claudins in several human epithelial tumors. For example, overexpression of claudin-3 and -4 was found in endometrial and ovarian cancer [4,5]. Furthermore, expression of claudin-3 and -4 in human ovarian epithelial cells is associated with increased invasiveness and cell survival *in vitro* [6]. Claudins have also been shown to modify invasion by the activation of matrix metalloproteinases (MMPs) [7]. On the other hand, claudin-4 is supposed to reduce the metastatic potential of pancreatic cancer [8]. Thus, although their exact function remains unclear, claudins may play a role in tissue remodeling, a process which is associated with several diseases including endometriosis.

Endometriosis is one of the most common gynecological diseases. Although endometriosis has been the subject of numerous scientific investigations, the pathogenesis is as yet unexplained. Irrespective of models for the pathogenesis of endometriosis (metaplastic and transplantation theory) invasiveness, cell differentiation and cell survival should have an essential role in endometriosis disease.

In the present work we analyzed the expression of several members of the claudin family by three complementary methods (microarray analysis, real-time polymerase chain reaction (PCR) and immunohistochemistry) in human endometrium and peritoneal endometriotic lesions. Claudin-3, -4 and -7 frequently displayed diminished expression in

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ectopic endometrium, as indicated by all three methods. The downregulation of various members of the claudin family may contribute to endometrial cell detachment and increase the number of cells invading pelvic organs.

Methods

Materials

Endometrial biopsies were taken from consecutive patients undergoing hysterectomy, dilation and curettage, and other procedures for benign gynecological diseases. All peritoneal biopsy specimens were taken from the lateral abdominal wall. The presence of endometriosis was subsequently confirmed histologically and unaffected normal peritoneum was macroscopically removed. All samples were quickly frozen and stored in liquid nitrogen. Characteristics of the samples are given in Table I. The study was approved by the local ethics committee. For suppliers of antibodies see the Immunohistochemistry section.

Microarray analysis

RNA preparation and microarray analysis using the Affymetrix Human Genome U133A GeneChip platform (Affymetrix, Inc., Santa Clara, CA, USA) containing 22 283 probes were performed as described elsewhere [9]. Hybridization intensity data were automatically acquired and processed using the Affymetrix Microarray Suite 5.0 program. The expression level of each gene was determined by calculating the average differences in intensity (perfect match–mismatch) between its probe pairs. Scans were rejected if the scaling factor exceeded 2 or a 'chip surface scan' revealed scratches, specks or gradients affecting the overall data quality (Refiner; GeneData, Inc., Basle, Switzerland).

The expression data were subsequently analyzed using the Cluster and Treeview software package [10]. Prior to cluster analysis, gene chip expression values were adjusted by log transformation and median centering of the gene chips. Hierarchical gene clustering was carried out using the Pearson correlation as distance metric and average linkage clustering.

Analyses of published datasets

Affymetrix gene expression data of endometrial tissues from the studies of Burney and colleagues [11] ($n = 37$, GEO database entry GSE6364) and Hever and associates [12] ($n = 251$, GEO database entry GSE7305 and GSE3707) were downloaded from the NCBI Gene Expression Omnibus database at http://www.ncbi.nlm.nih.gov/geo/. These independent datasets were used for verification, for analyses of claudin expression during the different phases of the endometrial cycle and to analyze the

Table I. Characteristics of the samples.

Microarray analysis – eutopic endometrium ($n = 3$)	Age of patients (years)	43.7 ± 2.3
	Intraepithelial cervical dysplasia	1
	Uterine fibroids	2
Microarray analysis – ectopic endometrium ($n = 3$)	Age of patients (years)	35.0 ± 2.0
	Endometriosis rARSM I/II	2
	Endometriosis rARSM III/IV	1
Microarray analysis – peritoneum ($n = 3$)	Age of patients (years)	40.7 ± 5.9
	Uterine fibroids	2
	Sterility	1
Immunohistochemistry – eutopic endometrium ($n = 22$)	Age of patients (years)	41.2 ± 6.6
	Intraepithelial cervical dysplasia	3
	Uterine fibroids	10
	Endometriosis rARSM I/II	4
	Adenomyosis	3
	Adenomyosis and fibroids	1
	Endometriosis and fibroids	1
	Proliferative phase	12
	Secretory phase	6
	Oral contraceptives	4
Immunohistochemistry – ectopic endometrium ($n = 22$)	Age of patients (years)	33.8 ± 6.3
	Endometriosis rARSM I/II	18
	Endometriosis rARSM III/IV	4
	Proliferative phase	13
	Secretory phase	1
	Oral contraceptives	5
	n.d. (hysterectomy in history)	3
Real-time PCR – eutopic endometrium ($n = 7$)	Age of patients (years)	41.3 ± 8.8
	Intraepithelial cervical dysplasia	1
	Uterine fibroids	1
	Endometriosis rARSM III/IV	2
	Adenomyosis	2
	Pelvic pain	1
	Proliferative phase	2
	Secretory phase	4
	Oral contraceptives	1
Real-time PCR – ectopic endometrium ($n = 7$)	Age of patients (years)	31.4 ± 7.4
	Endometriosis rARSM I/II	4
	Endometriosis rARSM III/IV	3
	Proliferative phase	1
	Secretory phase	0
	Oral contraceptives	5
	n.d.	1

PCR, polymerase chain reaction; rARSM: revised American Society for Reproductive Medicine score; n.d., not determined.

transcriptional profile of claudins among several human normal tissues in more detail.

Real-time polymerase chain reaction analysis

Real-time PCR analysis was carried out on samples from eutopic and ectopic endometrium ($n = 14$). Sample characteristics are given in Table I. Total RNA extraction from frozen tissues and real-time PCR analyses were performed as described previously [13] using the ABI 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA, USA). Briefly, cDNAs for all PCR analyses were generated by random primed reverse transcription (Wipeout cDNA Synthesis Kit; Qiagen, Valencia, CA, USA). Removal of genomic DNA was confirmed by reverse transcription negative PCR reactions. PCR reactions were carried out in accordance with the manufacturer's protocols (PE-Applied Biosystems). TaqMan Assays corresponding to the region of the Affymetrix claudin probe sets were applied to measure claudin-1, -3, -4, -5 and -7 mRNA. VIC-fluorophore-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan probes served as internal quantification markers in multiplex PCR reactions. Each quantification was reproduced three times and normalized by GAPDH using the Δc_t method.

Primer sequences were as follows.

- cldn1_up1: 5′-TAT TTC TTC TTG CAG GTC TGG C-3′
- cldn1_low1: 5′-CTG ACC AAA TTC GTA CCT GGC-3′
- cldn3_up1: 5′-CCC ACG CGA GAA GAA GTAC AC-3′
- cldn3_low1: 5′-CCC TGC GTC TGT CCC TTA GAC-3′
- cldn4_up1: 5′-CCT AGC CCT GCA TGG AGT CTC-3′
- cldn4_low1: 5′-CAC GCC TGT AAT CAC AGC TAC-3′
- cldn5_up2: 5′-CCT GTG AAG ATT GAG AGC TGC C-3′
- cldn5_low2: 5′-CGA AGC AGC CAA TCC GTG CGC-3′
- cldn7_up3: 5′-CAT CGT GGC AGG TCT TGC CGC-3′
- cldn7_low3: 5′-CAA ACT CAT ACT TAA TGT TGG-3′

Immunohistochemistry

Paraffin sections (2 μm) were mounted on Superfrost Plus slides, dewaxed in xylene and rehydrated through graduated ethanol to water. Antigens were retrieved by microwaving sections in 10 mM citrate buffer (pH 6.0) for 20 min at 800 W. Blocking was performed using antibody dilution buffer (DCS-Diagnostics, Hamburg, Germany) at room temperature for 15 min. Subsequently, antibodies were diluted 1:100 individually in this buffer. Sections were incubated with antibodies for 1 h at room temperature. For negative controls, the primary antibodies were replaced with phosphate-buffered saline. For secondary antibody incubations and detection, the Dako REAL Detection System Alkaline Phosphatase/RED (Dako, Glostrup, Denmark) was used following the protocol of the supplier and sections were counterstained with Mayer's hematoxylin. Polyclonal antibodies directed against different claudins (claudin-1, cat.# RB-9209; claudin-3, cat.# RB-9251; claudin-4, cat.# RB-9266; claudin-5, cat.# RB-9243; claudin-7, cat.# RB-10284) were obtained from Medac (Hamburg, Germany). Secondary goat anti-rabbit antibody (FAST-RED) was purchased from Dianova (Hamburg, Germany).

Statistical analyses

All reported p values are two-sided; p values of less than 0.05 were considered to indicate a significant result. The Mann–Whitney test was used to compare expression values between different tissues. All analyses were performed using the SPSS 11.0 package (SPSS Inc., Chicago, IL, USA).

Results

Detection of differential expression of claudins in endometriosis by microarray analysis

In human endometrium, the role of the claudin tight junction proteins is not well characterized. Because tight junctions represent one mode of cell-to-cell adhesion, proteins of the claudin family may be involved in various pathological disorders including the process of endometrial cell detachment and invasion of pelvic organs. Alteration of claudin expression has been demonstrated in patients with hyperplastic and malignant endometrial tissue [4]. To gain further insight into the transcriptional changes that take place before and after the development of endometriosis, we carried out microarray analysis of endometrial tissue, normal peritoneum and peritoneal endometriosis lesions (see Methods section). Using cluster analysis, we identified several candidate marker genes indicated to be specifically up- and downregulated in endometriosis tissues [14]. The principal plausibility of this approach was validated by the identification of MMP-9 from these microarray analyses, which has been previously described to be elevated in expression in endometriosis [15]. Among the identified genes, we detected several members of the claudin tight junction protein family, which showed an altered expression

in ectopic endometrium (Table II). While claudin-3, -4 and -7 were found at diminished levels in endometriotic lesions, claudin-1 displayed a 5.47-fold higher expression in ectopic in comparison with eutopic endometrium. Also claudin-5 was 2.97-fold higher expressed in samples of ectopic endometrium (Table II). Several other claudins (claudin-6, -8, -9, -11, -14, -16, -17, -18) were not expressed in either endometriotic tissue type or peritoneum (data not shown). Since our microarray profiling encompassed only a limited number of samples, we analyzed two additional published microarray datasets of Burney [11], Hever [12] and co-workers, which were downloaded from the Gene Expression Omnibus database (datasets GSE7307, GSE7305 and GSE6364). In agreement with our own microarray analysis, the relative expression levels of claudin-3, -4 and -7 in the eutopic endometrium samples were found to be higher than those of claudin-1 and -5 in all three datasets and clearly distinct from the claudin expression pattern of the ectopic endometrium (Figure 1).

Validation of microarray data by real-time polymerase chain reaction

We next carried out quantitative real-time PCR analysis as a validation of claudin expression among additional tissue samples. Figure 2 gives the results of these analyses. In line with microarray results we observed a significant higher mRNA expression for claudin-5 in peritoneal endometriosis tissue ($n = 7$) when compared with eutopic endometrium ($n = 7$; $p = 0.035$, Mann–Whitney test using Δc_t values as compared with GAPDH). However, there was no difference for claudin-1 and claudin-4 expression between the samples ($p = 0.85$ and $p = 0.34$, respectively). While there was a trend for a reduced expression of both claudin-3 and -7 in endometriosis tissue, this was not significant among the relatively small number of samples ($p = 0.16$ and $p = 0.23$, respectively). The failure to validate the expression differences of claudin-1 and -4 may be explained by a phase-specific regulation.

Table II. Expression differences of claudins in ectopic and eutopic endometrium and peritoneum based on microarray analysis.

		Ectopic vs. eutopic endometrium		Peritoneum vs. eutopic endometrium	
Gene	Affymetrix ProbeSet	Fold difference	SD	Fold difference	SD
Claudin-1	218182_s_at	5.47	0.34	1.81	0.11
Claudin-3	203954_x_at	0.11	0.02	0.16	0.02
Claudin-4	201428_at	0.32	0.06	0.05	0.01
Claudin-5	204482_at	2.97	0.54	0.29	0.05
Claudin-7	202790_at	0.32	0.07	0.37	0.08

SD, standard deviation.

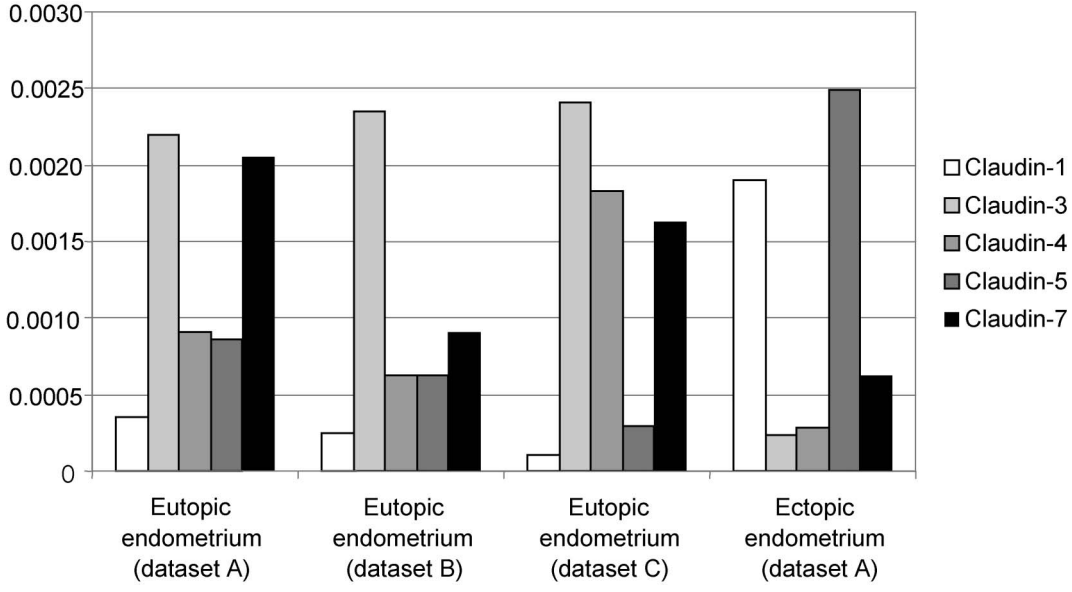

Figure 1. Microarray analysis of claudin expression. Bar chart giving the mean expression of different claudins in endometrium samples from the different datasets (dataset A, present study; dataset B, GSE7307; dataset C, GSE6364).

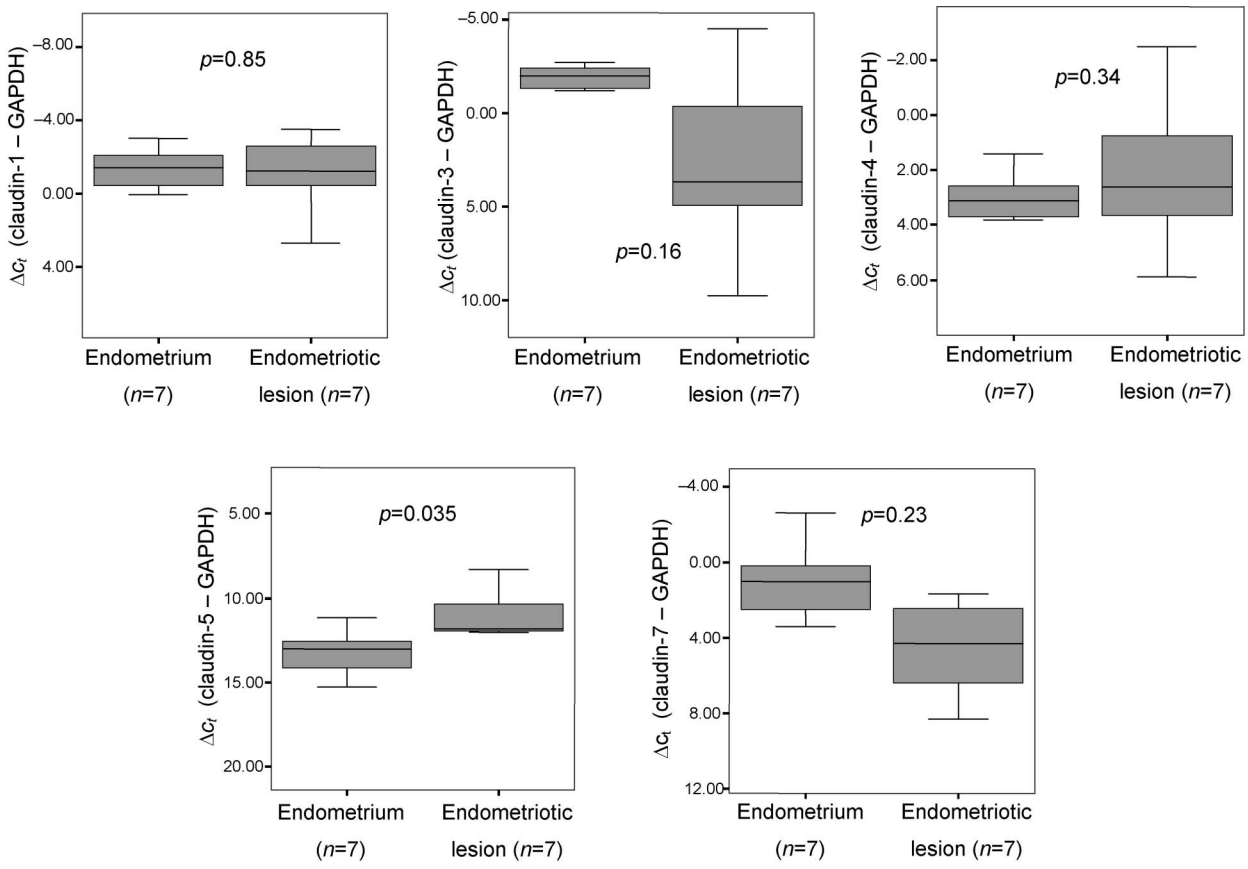

Figure 2. Real-time polymerase chain reaction (PCR) analysis of claudin expression. Analysis of the mRNA expression of claudin-1, -3, -4, -5 and -7 in samples of eutopic ($n = 7$) and ectopic ($n = 7$) endometrium. Box plots of the Δc_t values from quantitative real-time PCR analyses (normalized to VIC-fluorophore-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) are given.

Analysis of menstrual cycle-dependent expression of claudins mRNA

Since claudin expression may vary across the various phases of the menstrual cycle we used the published microarray datasets of Burney and co-workers [11] to analyze the transcriptional regulation in more detail. This endometrial microarray dataset consists of 11 samples from the proliferative phase (PE), nine samples of the early secretory phase (ESE) and 17 specimens of the mid-secretory stage (MSE). The analysis revealed a strong phase-specific regulation of claudin-3, -4 and -7 with enhanced expression during MSE, whereas no menstrual cycle dependency was detectable for claudin-1 and -5 (Figure 3).

Immunohistochemistry analysis identifies glandular epithelial cells as a major source of claudin expression

Immunostaining positive for claudin-1, -3, -4, -5 and -7 was observed in the eutopic endometrial glands of almost all of the specimens investigated (Table III). In most cases, membrane-bound staining was stronger in the apical and only in some cases in the basal cell borders, but it could also occur circumferentially. The staining pattern within each specimen was often very inhomogeneous – negative glands as well as weakly or strongly staining glands could all be present simultaneously. For the analysis shown in Table III the staining of epithelial cells in the samples was scored in three categories (strong, moderate and weak). For categorization of a tissue sample we analyzed at least 500 glandular cells and used the strongest staining observed in at least 20% of the cells. Using this scoring method the expression of claudin-1, -3, -4, -5 and -7 in either eutopic or ectopic endometrium showed no dependence on the phase of the menstrual cycle, the intake of oral contraceptives, or the patient's diagnosis (data not shown).

In ectopic endometrium, a tendency toward weaker expression of all of the claudins in the epithelial cells could be identified (Table IV and Figure 4), which is in contrast to the data from microarray and real-time PCR suggesting a stronger expression of claudin-1 and claudin-5 in ectopic endometrium. However, in almost all of the samples studied, the vessels showed moderate to strong expression of claudin-5 which was not observed for the other claudins (data not shown).

Mesothelial cells of peritoneal endometriosis showed moderate to strong staining with claudin-1,

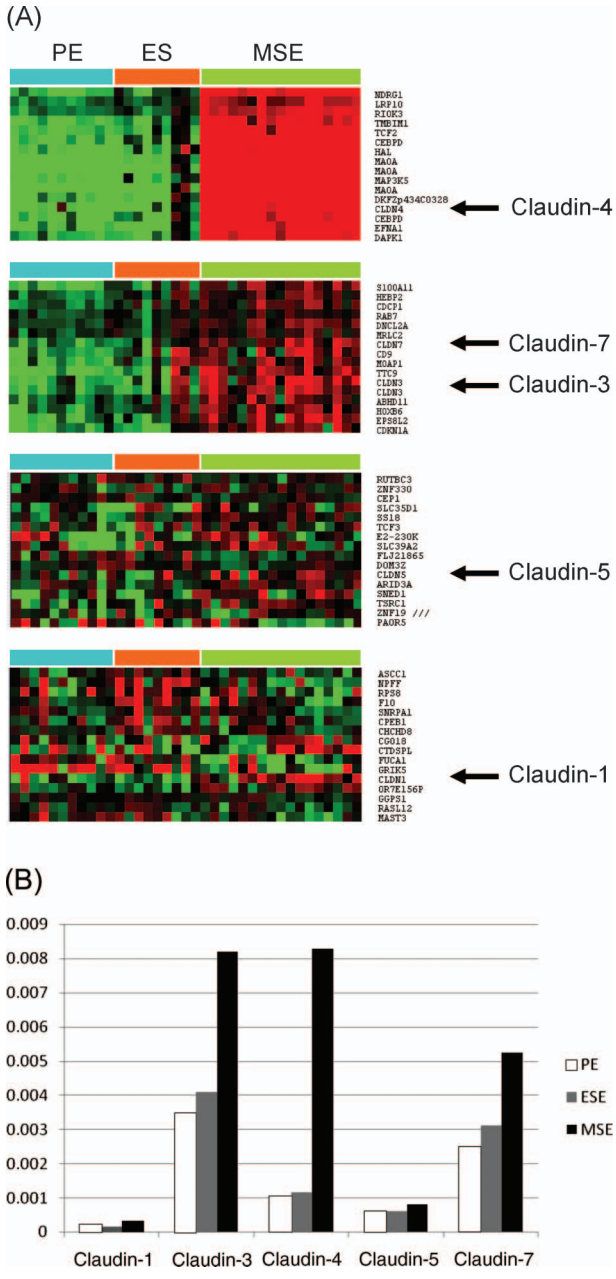

Figure 3. Dependency of claudin expression on the menstrual cycle phase. (A) Endometrium samples from the microarray dataset of Burney and colleagues [11] (GSE6364) were used to analyze expression of claudins during the menstrual cycle. Microarray data were log transformed and genes were subsequently median centered to detect relative transcriptional regulation (red, above median; green, below median). Genes correlating with the different claudins after hierarchical clustering are shown. Patients are stratified according to menstrual cycle phase: PE, proliferative phase; ESE, early secretory phase; MSE, mid-secretory phase. As shown, claudin-3, -4 and -7 are strongly associated with menstrual cycle phase. (B) Mean expression of different claudins in endometrium samples are given from patients stratified as in (A).

while the other claudins showed no reaction or only a weak one occasionally.

In summary, on the basis of the immunohistochemistry results in both eutopic and ectopic endometrium, the expression of claudin-3, claudin-4 and claudin-7 is basically the result of expression in the glandular cells. By contrast, claudin-5 is also found to a substantial extent in the vascular endothelial cells and claudin-1 is found in the mesothelial cells.

Discussion

Relatively sparse data have been available to date regarding the role of the claudins as tight junction proteins in the endometrium, and the data are also partly inconsistent [16,17]. Sobel and colleagues [1] reported menstrual cycle-dependent upregulation of claudin-4 in PCR, while significant changes in expression were not observed for claudin-1, -3, -5 or -7. In agreement with these results, Pan and associates [4] also observed upregulation of claudin-3 and -4 during the secretion phase, in contrast to the proliferation phase. Since according to our analyses increases in claudin-3, -4,and -7 mRNA in microarray measurements are seen only in the mid-secretory phase, these differences could be explained by discrepancies in the assumed and the real menstrual cycle phase. However, the physiological function of claudins and their potentially hormone-dependent regulation are still unclear at present. While Pan and co-workers were also able to demonstrate the differences in claudin expression described above using immunohistochemistry, no dependency of the staining pattern or immunohistochemistry score on the menstrual cycle phase or hormone intake was observed in the present study. In the study by Pan's group, approximately 50% of the endometrial samples examined were negative while the others were weakly stained. The strong immunostaining observed on average in our samples may perhaps make it more difficult to recognize differences in protein expression.

Both in the microarray analysis and to some extent in PCR, stronger expression of claudin-1 and -5 and reduced expression of claudin-3, -4 and -7 were observed in ectopic endometrium in comparison with eutopic endometrium. On immunohistochemistry, by contrast, the epithelial endometrial cells in the ectopic endometrium showed an insignificantly reduced staining score for all of the claudins investigated. The increased expression of claudin-1 and -5 in ectopic endometrium on microarray and PCR can be explained by the strong expression of claudin-1 in the mesothelial cells in the peritoneal endometriosis samples and the numerous vessels expressing claudin-5 identified. The relatively small number of samples in the microarray analysis and the widely varying proportions of accompanying mesothelial cells, depending on the sample being examined, must be regarded as the reason for the inconsistent results between PCR and microarray analysis.

Table III. Immunohistochemical staining of glandular cells in eutopic endometrium.

Staining pattern	Claudin-1	Claudin-3	Claudin-4	Claudin-5	Claudin-7
Negative	None	None	None	1/21 (5)	None
Weak	4/22 (18)	3/22 (14)	3/20 (15)	5/21 (24)	3/19 (16)
Moderate	13/22 (59)	10/22 (45)	8/20 (40)	10/21 (48)	8/19 (42)
Strong	5/22 (23)	9/22 (41)	9/20 (45)	5/21 (24)	8/19 (42)

Data are *n*/*N* (%).

Table IV. Immunohistochemical staining of glandular cells in ectopic endometrium.

Staining pattern	Claudin-1	Claudin-3	Claudin-4	Claudin-5	Claudin-7
Negative	1/17 (6)	1/19 (5)	3/20 (15)	2/19 (10)	2/21 (10)
Weak	9/17 (53)	6/19 (32)	9/20 (45)	9/19 (47)	8/21 (38)
Moderate	5/17 (29)	5/19 (26)	5/20 (25)	6/19 (32)	3/21 (14)
Strong	2/17 (12)	8/19 (42)	3/20 (15)	2/19 (10)	8/21 (38)

Data are *n*/*N* (%).

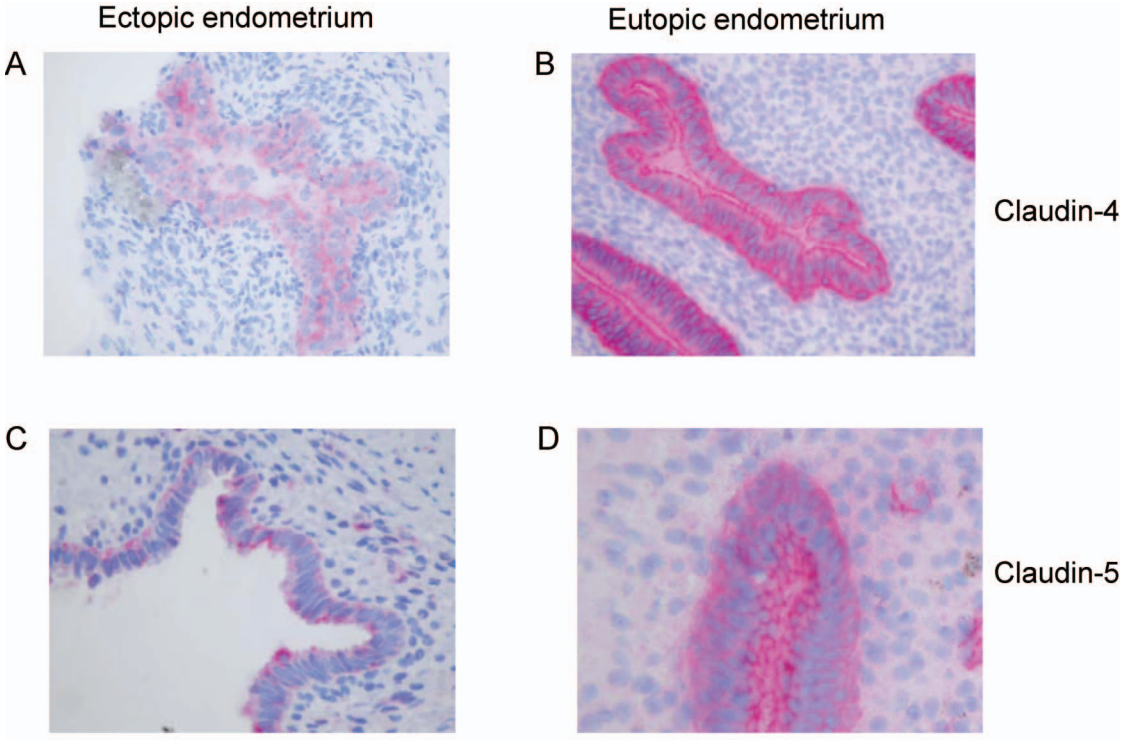

Figure 4. Immunohistochemical analysis of claudin expression. Shown are representative stainings (red) of claudin-4 (A and B) and claudin-5 (C and D) in ectopic (A and C) and eutopic (B and D) endometrium, respectively. Magnification: 25× in A and B; 40× in C and D; counterstain: Mayer's hematoxylin (blue).

Since atypical hyperplasias are associated with increased expression claudin-3 and -4 it has been suspected that these claudins may play a role in the development of endometrioid endometrial carcinoma. In addition, infiltration of the myometrium correlates with the expression of claudin-3 and -4 [4]. Consistent with this view is the observation that invasiveness of ovarian epithelial and ovarian cancer cells is increased by claudin-3 and -4. However, the precise relationship between claudin expression and cancer initiation is as yet unclear. Changes in the tight junctions, with subsequent changes in cell adhesion, are thought to be involved. While the upregulation of claudin-3 and -4 plays a role in type 1 endometrial and ovarian carcinoma, and upregulation of claudin-1 plays a role in type 2 endometrial carcinoma, the invasiveness and metastatic capacity of other tumors are associated with downregulation of claudins [1,4,6]. In pancreatic carcinoma cells, for example, the expression of claudin-4 leads to reduced

invasiveness *in vitro*, and in breast carcinoma, reduced expression of claudin-7 leads to increased cellular discohesion [8,18]. These discrepancies are thought to be caused by extremely tissue-specific functioning of the claudins. In this context, the reduced expression of the claudins detected in ectopic endometrium – the origin of which must even still be regarded as unexplained – may well be causally connected to its invasive phenotype.

In summary, the present study provides evidence of reduced expression of claudins in peritoneal endometriosis lesions in comparison with eutopic endometrium, and this may be associated with the invasive characteristics of endometriosis. In the context of the inverse expression of claudins in ectopic endometrium in comparison with endometrioid endometrial carcinoma, the tissue-specific role postulated for the claudins in tumor development and cell adhesion/invasiveness once again raises the topic of whether endometriosis originates in eutopic endometrium.

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