Cold-shock domain family member YB-1 expression in endometrium and endometriosis

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Introduction

Endometriosis (Eo) is a chronic inflammatory and progressive disease with a high prevalence. It is defined by the presence of endometrium-like glands and stroma outside the uterus, commonly observed on visceral and peritoneal surfaces throughout the female abdominal cavity. Besides the association with intense pain and infertility, Eo is one of the most problematic and most frequent gynecological disorders, affecting 6–10% of women of reproductive age (Eskenazi and Warner, 1997). Although it is considered a benign disease, Eo has several similarities with malignant tumors (Witz et al., 2003). Strong dissemination and deep invasion in tissues such as peritoneum, ovary and intestine, increased angiogenesis and high recurrence risks with a chronic course are typical for this disease. Remarkably, Eo is also characterized by an inflammatory process in the abdomen involving immune cells which have changed their actual function. The peritoneal fluid of affected patients contains a high number of activated macrophages that secrete growth factors and cytokines.

The progression of Eo, and the often associated infertility, could be caused by high levels of cytokines in the peritoneal fluid and serum of affected patients (Badawy et al., 1984; Oral et al., 1996; Ho et al., 1997; Iwabe et al., 2000; Gazvani and Templeton, 2002; Pizzo et al., 2002). Chemokines, especially RANTES (regulated upon activation, normal T-cell expressed and secreted chemokine) expression and apoptosis (ELISA-based assay).

BACKGROUND: The Y-box-binding protein (YB-1) is described as a potential oncogene highly expressed in tumors and associated with increased cell survival, proliferation, migration and anti-apoptotic signaling. The aim of our study was to examine the expression and role of YB-1 in human endometriosis (Eo) and its association with cell survival, proliferation and invasion.

METHODS: We analyzed the gene and protein expression levels of YB-1 by quantitative real-time RT–PCR and immunoassays, respectively, in peritoneal macrophages, ovarian endometrioma and eutopic endometrial tissues/cells derived from women with (n = 120) and without (n = 91) Eo. We also evaluated the functional consequences of YB-1 knockdown in the Z12 Eo cell line by measuring cell proliferation [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid cell proliferation assay], invasion (Matrigel invasion assay) and spontaneous and tumour necrosis factor (TNFα)-induced RANTES (regulated upon activation, normal T-cell expressed and secreted chemokine) expression and apoptosis (ELISA-based assay).

RESULTS: YB-1 gene and protein expression was statistically significantly higher in ovarian lesions, eutopic endometrium and peritoneal macrophages of patients with Eo in comparison with the control group. Interestingly, the strongest YB-1 expression was observed in the epithelial compartment of endometrial tissues. In the Z12 cell line, YB-1 knockdown resulted in significant cell growth inhibitory effects including reduced cell proliferation and increased rates of spontaneous and TNFα-induced apoptosis. Significantly, higher RANTES expression and decreased cell invasion in vitro were also associated with YB-1 inactivation.

CONCLUSION: High YB-1 expression could have an impact on the development and progression of Eo. This study suggests the role of YB-1 as a potential therapeutic target for Eo patients.

Key words: endometriosis / endometrium / YB-1 / apoptosis / inflammation
normal T-cell expressed and secreted chemokine) are necessary for the recruitment of different leukocyte subtypes to places of inflammatory events and endometriotic lesions (Rossi and Zlotnik, 2000). About 70% of the chemotactic monocyte activity in the peritoneal fluid of women with Eo is mediated by RANTES (Hornung et al., 2001).

High epithelial atypia in endometriotic tissue may represent premalignant lesions which could turn into cancer. In about 0.5% of the patients, ovarian Eo turns into a histologically endometrial type of ovarian cancer (Czernobilsky et al., 1970; Oral et al., 2003). Particularly in ovarian cancer, YB-1 is described as an oncogene and its impact as a predictive marker is emphasized (Bargou et al., 1997; Shibahara et al., 2001; Gimenez-Bonafe et al., 2004; Fujita et al., 2005; Oda et al., 2007; Wu et al., 2007; Fuji et al., 2008a). The Y-box-binding protein (YB-1), an evolutionary conserved 48 kDa protein, belongs to the superfamly of cold-shock domain proteins with pleiotropic biological functions. Eu-karyotic YB proteins are involved in the regulation of DNA transcription and repair, and in translational control of protein synthesis as well as in cellular responses to a wide variety of stressors (Matsumoto and Wolff, 1998; Kohno et al., 2003; Lage et al., 2008). The YB-1 protein is highly expressed in a number of malignant diseases, and it seems to promote tumor growth and multi-drug resistance (Chatterjee et al., 2008) through the induction of specific growth factors (Bargou et al., 1997; Wu et al., 2006; Fuji et al., 2008b; Habibi et al., 2008).

Based on the multiple biological functions of YB-1 and its close association with tumorigenesis, we investigated YB-1 expression in human endometrium, ovarian Eo and peritoneal macrophages.

Materials and Methods

Patients, samples and cell line

Endometrium, endometriotic tissue and peritoneal fluid were obtained from patients with histologically diagnosed Eo (n = 120, age distribution: 35.7 ± 6.7 years) undergoing laparoscopy at the Department of Obstetrics and Gynecology, University of Lübeck, Germany. Patients in the control (Co) group (n = 91, age distribution: 34.6 ± 11.7 years) underwent laparoscopy for uterine myoma (n = 71) or sterilization (n = 20) without any sign of Eo. Endometrial tissues and peritoneal fluids (from Eo patients and Cos) were always obtained in the mid-proliferative phase of the menstrual cycle under sterile conditions. Eo and Co patients were not receiving hormone therapy at the time of the study or in the previous 3 months, and they did not have clinical signs of rheumatologic or immunologic diseases. Eo was staged intra-operatively according to the previous 3 months, and they did not have clinical signs of rheumatologic or immunologic diseases. Eo was staged intra-operatively according to the previous 3 months, and they did not have clinical signs of rheumatologic or immunologic diseases.

Isolation of peritoneal macrophages

Peritoneal fluid of women with (n = 38) and without (n = 17) Eo was centrifuged for 5 min at 1500g. The supernatant was collected, and the cell pellet was resuspended in 2 ml phosphate-buffered saline (PBS). After 20 min of gradient centrifugation with lymphocyte separation medium (PAA) at a maximum of 700 g, the cell ring between PBS and separation medium was extracted and resuspended in PBS. After repeated centrifugation at 1500g, the cell pellet was stored at −80°C until needed. Macrophages were confirmed by positive staining for CD68.

Extraction and purification of mRNA from tissues and cells

In endometrial tissue samples, total RNA isolation was performed using the RNAsin kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Total RNA from cultured cells and peritoneal macrophages was extracted using Trizol solution (Invitrogen) according to Chomczynski and Sacchi (1987). DNase I treatment (Invitrogen) was performed before cDNA synthesis. The quality and concentration of the extracted total RNA was verified using the Biophotometer Plus (Dynatech Laboratories, VA, Canada).

Quantitative real-time RT-PCR

Polyadenylated mRNA was reverse transcribed using oligo (deoxythymidine) primers (Invitrogen). Quantitative PCR assay was performed with the Engine Opticon 2 System (MJ Research, Waltham, MA, USA) using 2× Platinum SYBRGreen qPCR SuperMix (Invitrogen) and specific oligonucleotide primers for YB-1 (forward, 5′-GGGTGCGAGGAGCAAGGTA-3′; reverse, 5′-TCCGATCATGGTCTTCCTCTC-3′) as well as HPRT genes (forward, 5′-CCGGCAGGTGATTATTGAT-3′; reverse, 5′-CCACGCACAGCAAGAAATTATTA-3′) (Metabion, Martinsried, Germany). High-performance liquid chromatography–H₂O and template without reverse transcriptase were used as negative controls. Primer efficiency was confirmed by conventional RT-PCR and PCR products were visualized on 2% agarose gels (3% NuSieve GTG, 1% Sea Kem GTG, FMC Bioproducts, Rockland, ME, USA) and stained by ethidium bromide (Life Technologies, Gaithersburg, MD, USA).

Western blot analysis

Briefly, endometrial tissue lysates and lysates of peritoneal macrophages from Eo and Co patients were subjected to SDS-PAGE, transferred to nitrocellulose membrane and blocked with 5% non-fat dry milk prior to incubation with polyclonal rabbit-anti-YB-1 antibody (Antikörper Online, Aachen, Germany). Bound antibody was detected with horseradish peroxidase-conjugated goat-anti-mouse-IgG (VWR, Darmstadt, Germany) and enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). Monoclonal mouse-anti-β-actin was used as a loading control (Sigma, Steinheim, Germany).

Immunocytochemistry

Primary cultures of epithelial and stromal cells (n = 12) were cultivated on chamber slides (NUNC, Wiesbaden, Germany) at 37°C and 5% CO₂ and fixed for 10 min with methanol/acetone (70/30) at −20°C. To increase permeability, the cells were treated with 0.5% saponin (Fluka, Steinheim, Germany) for 30 min at 37°C. After blocking with 10% goat serum (Sigma) at room temperature, the slides were incubated with...
monoclonal mouse anti-hYB-1 primary antibody (Antikörper Online) and monoclonal mouse anti-hCK18 antibody (Imgenex, San Diego, CA, USA), respectively, over night at 4°C. All other steps were performed at room temperature. Subsequently, slides were incubated with biotinylated goat-anti-mouse IgG (Vector, Burlingame, CA, USA) secondary antibody for 30 min, followed by incubation with streptavidin-linked alkaline phosphatase for 20 min and finally with fast red/naphthol phosphate (Dianova, Hamburg, Germany) for 10 min. After nuclear staining with Mayer’s Hämalun (Merck, Darmstadt, Germany), slides were covered with Faramount Aqueous Mounting Medium (DAKO, Hamburg, Germany). Negative controls were performed with PBS instead of the primary antibody. Pictures were taken with the Axiosvert 135 M (Zeiss, Oberkochem, Germany) at 200–320-fold magnification.

Immunohistochemistry
Eutopic endometrial tissue (n = 19) and ovarian endometriotic lesion samples (n = 13) were formalin fixed and paraffin embedded (FFPE). Superfrost PLUS slides (Menzel, Brauschweig, Germany) containing 4-μm FFPE tissue sections were deparaffinized and treated with antigen unmasking solution (Vector) and 0.5% saponin (Sigma). After blocking with normal goat serum for 30 min, slides were incubated with monoclonal mouse anti-hYB-1 antibody (Antikörper Online) over night at 4°C.

Subsequently, the slides were incubated with biotinylated goat-anti-mouse-IgG, followed by alkaline phosphatase-linked streptavidin incubation. Reactions were developed with fast red/naphthol phosphate and slides were counter stained with Mayer’s Hämalun and cover slipped using the Faramount Aqueous Mounting Medium. Immunohistochemical reactions were microscopically analyzed at 200–320-fold magnification. Negative controls were included in all assays in accordance with the manufacturer’s recommendations.

YB-1 knockdown in epithelial endometriotic Z12 cell line
Z12 cells were cultivated in well plates as described (Zeitvogel et al., 2001) and transfected with 10-nM Hs YBX-1 HP validated siRNA (sense strand, 5′-GGCGGAGGUGUCCACCUUATT-3′; Qiagen) using a INTERFERin™ transfection reagent (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions. Non-mammalian target siRNA (sense strand, 5′-UACCGACUAAAACACAUCA-3′; Dharmaco/Thermo Fisher Scientific, Schwerte, Germany) was used as negative control. Additional experiments included 3′-Alexa Fluor 488 modified Hs YBX1 HP validated siRNA, AllStars Hs cell death control siRNA (Qiagen) and mock-transfected cells (transfection media only). YB-1 protein expression was analyzed 1–5 days after transfection in knockdown and control cells by western blot analysis as described above.

Cell proliferation assay
The viability of YB-1 siRNA transfected Z12 cells was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) cell proliferation assay (Promega), according to the manufacturer’s instructions. The proliferation rate was determined spectrophotometrically at 560 nm (reference wavelength of 650 nm) using the Dynatech MRX photometer (Dynatech Laboratories).

Apoptosis assay
Spontaneous and tumour necrosis factor alpha (TNFα)-induced (20 ng/ml; 40 ng/ml) apoptosis was analyzed using cell death detection ELISA Plus (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer’s instructions and following necrotic cell fraction removal. The absorbance of each well was photometrically measured at 405 nm (reference wavelength of 490 nm) using the Dynatech MRX photometer (Dynatech Laboratories).

RANTES expression analysis
CCL5/RANTES expression was analyzed in TNFα-stimulated (100 ng/ml) and unstimulated YB-1 siRNA transfected and control cells. The cell culture supernatant was collected and RANTES levels were determined using the QuantiKine Human CCL5/RANTES ELISA (R&D Systems, Abingdon, UK) according to the manufacturer’s protocol. The optical density of each well was photometrically detected using the Dynatech MRX photometer (Dynatech Laboratories) set to 450 nm (reference wavelength 490 nm).

Invasion assay
Cell invasion in vitro was evaluated using a double-filter assay (Erkell and Schirmacher, 1988). Briefly, thin gels consisting of a fibrin-casein mixture and Matrigel (BD Bioscience, Heidelberg, Germany) basement membrane extract were prepared between two filters (a bottom supporting 5-μm pore nitrocellulose filter and a top 5-μm pore matrigel-coated polycarbonate filter, Millipore, Ireland) in 24-well plates. YB-1 siRNA transfected and control cells were added to well plates 72 h after transfection and incubated for 24 h at 37°C. Each filter set was stained with DAPI (Biotek, Bad, Friedrichshall, Germany) and absorbance was measured using the Synergy HT Fluorescence plate reader (Biotek, Bad Friedrichshall, Germany) at 460 nm (excitation wavelength 360 nm).

Statistical analysis
Differences between the groups were evaluated using Welch t-test, one-way ANOVA (with Bonferroni’s multiple comparison post-test or in combination with Student—Newman–Keuls test), Kruskall–Wallis or Wilcoxon tests and the results were considered statistically significant when analyses yielded P < 0.05. Statistical analyses were performed using Prism 4 for Windows (GraphPad Software, 2003, San Diego, CA, USA) and SPSS statistical software package for Microsoft Windows (version 14.0, SPSS Inc, Chicago, IL, USA).

Results
Analysis of YB-1 gene expression in endometrium and peritoneal macrophages
Quantitative RT–PCR analysis revealed a statistically significant increase in YB-1 gene expression in endometrium from women with Eo (1.63 ± 0.13 ratio YB-1/HPRT) in comparison with the Co endometrial tissues (1.00 ± 0.05 ratio YB-1/HPRT; P < 0.01) (Fig. 1A). Similarly, expression of YB-1 gene was statistically significantly higher in ovarian endometriotic lesions (2.01 ± 0.31 ratio YB-1/HPRT) in comparison with eutopic endometrium from women without Eo (1.00 ± 0.05 ratio YB-1/HPRT) (P < 0.001). Furthermore, we observed in this analysis that YB-1 gene expression was significantly higher in cultured epithelial cells (2.41 ± 0.77 ratio YB-1/HPRT) when compared with stromal cells (0.81 ± 0.16 ratio YB-1/HPRT) derived from endometrium of Eo patients (P < 0.05). The YB-1 expression in epithelial endometrial cells from women with Eo was also significantly higher in comparison with epithelial cells (0.9 ± 0.21 ratio YB-1/HPRT) and stromal cells (0.82 ± 0.22 ratio YB-1/HPRT) from Co endometrium (P < 0.05; Fig. 1B).

YB-1 gene expression analysis revealed similar results in peritoneal macrophages. Statistically significant higher YB-1 gene expression was
detected in peritoneal macrophages from patients with Eo (1.92 ± 0.38 ratio YB-1/HPRT) compared with peritoneal macrophages from women without Eo (1.00 ± 0.18 ratio YB-1/HPRT; P < 0.05; Fig. 1C).

**Analysis of YB-1 protein in endometrium, endometriotic lesions, ovarian lesions and peritoneal macrophages**

The results obtained by quantitative real-time PCR analysis could be confirmed on the protein level. Western blot analysis showed increased expression of YB-1 protein in the eutopic endometrial tissue samples from patients with Eo in comparison with Co endometria (Fig. 2A). Densitometry revealed statistically significantly higher expression of YB-1 in the endometrium of women with Eo (137.7 ± 22.57 ratio YB-1/β-actin) compared with Co samples (37.59 ± 11.39 ratio YB-1/β-actin; P = 0.002).

Furthermore, western blot analysis in peritoneal macrophages supported the results of the quantitative real-time PCR (Fig. 2B). Statistically significantly higher YB-1 protein expression was detected in macrophages of patients with Eo (385.4 ± 91.96 ratio YB-1/β-actin) in comparison with women without Eo (100.0 ± 49.41 ratio YB-1/β-actin; P = 0.0171). Strongest YB-1 protein levels were observed in peritoneal macrophages of patients with Eo in Stage III and recurrent cases.

The results from quantitative real-time RT–PCR and western blot analyses could also be confirmed using immunohistochemistry and immunocytochemistry. We particularly observed considerable expression of YB-1 in the epithelial compartments of endometrium of patients with Eo (12/12 cases) and of ovarian endometriotic (8/9 cases) samples. In contrast, no relevant YB-1 staining was detected in the stromal cells or in the epithelial or stromal compartments in tissues of women without Eo (n = 7) (Fig. 3).

Additionally, immunocytochemistry analysis revealed high protein expression of YB-1 in cultured endometrial epithelial cells derived from women with Eo (6/6 samples) compared with endometrial epithelial cells from women without Eo (n = 6; Fig. 4). No YB-1 staining was observed in stromal cells of eutopic endometrium from patients with (n = 6) or without (n = 6) Eo after 48 h of cell culture.

**Effects of YB-1 knockdown on cell proliferation, apoptosis, inflammatory response and invasion of endometriotic epithelial cell line**

The role of YB-1 in Eo pathogenesis was supported by the analysis of the effect of YB-1 siRNA on cell growth and apoptosis in endometriotic epithelial cells. Firstly, the efficacy of YB-1 siRNA knockdown in Z12 cells was confirmed by microscopic visualization of fluorescent Alexa labelled-YB-1 siRNA cell staining (data not shown). Subsequently, western blotting analysis showed that YB-1 protein expression was reduced to about 60% in YB-1 siRNA transfected cells at 72 and 96 h after transfection (Fig. 5A).

The effect of YB-1 siRNA on the growth of Z12 endometriotic cells is shown in Fig. 5B. We found that down-regulation of YB-1 expression caused significant cell growth inhibition in intracellular YB-1 siRNA transfected cell lines (84 ± 10.62) compared with Co (non-
targeting siRNA, 100 ± 10.58; Mock, 121 ± 15.72; untransfected cells, 159 ± 31.18; P < 0.001). In order to investigate whether the growth inhibitory effects of YB-1 siRNA are partially related to the induction of apoptosis, the effect of YB-1 siRNA on apoptotic cell death was examined using an ELISA-based assay. The results provided convincing data that down-regulation of YB-1 induces spontaneous (125 ± 18.43) and TNFα-stimulated (134 ± 12.15 and 135 ± 11.93 for 20 and 40 ng/ml, respectively) apoptosis in YB-1 knockdown cells (P < 0.005; Fig. 5C). Lower relevant apoptosis rates were observed in non-targeting siRNA, mock and untransfected cells.

Additionally, we evaluated the role of YB-1 in inflammatory response and invasion promotion in Eo. In TNFα-stimulated and unstimulated Z12 cells, we observed that YB-1 knockdown significantly increased the release of CCL5/RANTES (778 ± 9.25 and 118 ± 5.13, respectively) into the cell culture supernatant compared with non-targeting YB-1 transfected cell cultures (629 ± 75.4 and 103 ± 4.74, respectively; P ≤ 0.02; Fig. 5D). Although not statistically significant, YB-1 knockdown tended to suppress cell invasion in comparison with non-targeting siRNA transfected cells (Fig. 5E).

**Discussion**

In the present study, we demonstrated significantly higher gene and protein expression of YB-1 in endometrial and endometriotic cells and tissues as well as peritoneal macrophages from Eo patients. Interestingly, the highest expression of YB-1 was detected in endometrial epithelial cells. In endometriotic epithelial cells, YB-1 knockdown affected cell growth, survival and invasion.

The transcription factor YB-1 is described as an oncogenic protein which promotes tumor growth and chemotherapy resistance through induction of specific growth factors (Habibi et al., 2008; Takahashi et al., 2010). The nuclear YB-1 expression is closely associated with global drug resistance, malignant transformation and progression in a large number of human tumors (Chatterjee et al., 2008; Davies et al., 2011, in press). Furthermore, YB-1 levels are inversely correlated with the overall survival of patients and used as a prognostic marker in certain types of cancer (Kashiwhara et al., 2009).

To date, little is known about the contribution of YB-1 to the development and progression of Eo. As Eo is associated with inflammation, increased cell proliferation, adhesion procedures and, in rare cases, malignant transformation (Melin et al., 2006), YB-1 might play a pivotal role in the etiology and pathogenesis of the disease. In this study, we showed a potential connection between Eo and YB-1 expression in the endometrial tissues. We detected statistically significantly stronger expression of the transcription factor YB-1 in endometrium, endometriotic ovarian lesions and peritoneal macrophages of patients with Eo in comparison with women without Eo. Interestingly, the strongest YB-1 gene and protein expression levels were particularly observed in the epithelial compartments of the endometrial tissues from Eo patients.

As a member of the cold-shock family, YB-1 is involved in multiple biologic functions and is highly expressed in a number of malignant diseases, promoting tumor growth through the induction of specific growth factors, such as epidermal growth factor, transforming growth factor-beta and members of the matrix metalloproteinase (Bergquin et al., 2005) and MAPK (Jürchott et al., 2010) families. Our present study provides for the first time in vitro evidence that YB-1 could also be implicated with the markedly progressive characteristics of endometriotic lesions. Similar to findings in tumor cell lines (Shiota et al., 2008; Basaki et al., 2010), we show that YB-1 knockdown by targeting siRNA leads to a significant decrease in cell proliferation in epithelial endometriotic cells. Furthermore, the reduction of YB-1 expression caused a potent apoptotic effect suggesting that the growth inhibitory activity of YB-1 down-regulation may be partly attributed to increasing cell death. Indeed, the impact of YB-1 inhibition on apoptosis is well documented in several human cancer cell lines (Lasham...
et al., 2000; Homer et al., 2005; Lee et al., 2008), and YB-1 may be important for the development and survival of Eo as well. The disruption of host tissue architecture is certainly another important pathophysiologic characteristic of some endometriotic lesions (Zeitvogel et al., 2001). Recently, YB-1 has also been shown to drive cancer cell invasion (Astanehe et al., 2009; Evdokimova et al., 2009a). Our results from YB-1 knockdown experiments also showed YB-1 as a mediator of cell invasion in Z12 endometriotic epithelial cells. In fact, recent studies from Evdokimova et al. (2009a,b) have provided convincing evidence that up-regulation of YB-1 may affect epithelial–mesenchymal transition programming leading to the loss of epithelial proteins (f.e., E-cadherin) and properties, and, consequently, to the loss of deficient cell–cell contact control in invasive breast carcinoma cells. As a result of connecting YB-1 inhibition to suppressed endometriotic cell invasion in vitro, we speculate that YB-1 expression promotes an invasive phenotype in Eo by altering epithelial–mesenchymal interactions.

The statistically significantly stronger YB-1 gene and protein expression in peritoneal macrophages from patients with Eo compared with those without Eo could be associated with the inflammatory character of the disease. The peritoneal fluid of patients suffering from Eo contains large amounts of activated macrophages. They secrete cytokines and growth factors (Agic et al., 2006) which are associated with the progression of the disease. The chemotactic macrophage activity is mediated by RANTES (Hornung et al., 2001) and other cytokines. Increased levels of RANTES were found in the peritoneal fluid of women suffering from Eo and are positively associated with the stage of disease. Furthermore, RANTES mRNA and protein expression has been detected in eutopic endometrium, endometriotic lesions and cultured endometriotic stromal cells when induced by proinflammatory cytokines, such as TNFα, interferon gamma (IFNγ) and interleukin beta (Hornung et al., 2001). Indeed, it has been shown that YB-1 may act as a cell-type specific regulator for RANTES expression in macrophages (Krohn et al., 2007; Raffetseder et al., 2009). In addition, YB-1 is described as being secreted in the non-classical mode after inflammatory challenges and exerts mitogen and pro-migratory effects in inflammation (Frye et al., 2009). Secondly, extracellular YB-1 was found to be a ligand for Notch-3 receptors, activating the downstream signaling pathway. Therefore, it may be of particular relevance to inflammatory diseases (Rauen et al., 2009).

Figure 3 YB-1 protein expression pattern in endometrium (Eo, n = 12; Co, n = 7) and Eo (n = 9) by immunohistochemical analysis. For each sample, a negative control for YB-1 staining was performed (right column: Aa, Bb, Cc). (A) Significant YB-1 protein expression was detected in endometrium from a patient with Eo Stage III, predominantly in the epithelial compartment (magnification, ×100). (B) Strong YB-1 staining was also observed in the epithelial layer of ovarian Eo Stage IV (magnification, ×200). (C) No significant expression of YB-1 protein was detected in endometrial tissue from women without Eo (magnification, ×100).
On the other hand, YB-1 may also have anti-inflammatory functions in specific cell types (Inagaki et al., 2005; Dooley et al., 2006). In this study, we detected anti-inflammatory effects of YB-1 in endometriotic epithelial cells which showed significant higher RANTES expression after YB-1 knockdown. In fact, epithelial cells may exert a modulator role in local immune responses to maintain tissue integrity (Polito and Proud, 1998). Additionally, we previously demonstrated in vitro the discrepant RANTES expression between endometrial stromal and epithelial cells by showing that low levels of RANTES transcripts and protein were observed in TNFα and IFNγ-stimulated epithelial cell cultures (Hornung et al., 1997). Thus, it is also possible that, at least in the epithelial compartment, YB-1 has a regulatory effect during Eo development by inhibiting specific pro-inflammatory signals and thereby supporting survival of ectopic endometrial epithelia.

Although the validation of the Z12 cell line as an in vitro model of Eo has only been partially performed, several studies have shown that these cells may act similarly to endometriotic cells. Indeed, the human immortalized endometriotic epithelial Z12 cell line was established from active endometriotic lesions by in situ electroporation of endometriotic cells with a plasmid containing the SV40 virus (Zeitvogel et al., 2001). This cell line retains the phenotypic characteristics as well as several in vivo properties of Eo, and gene expression analyses have

**Figure 4** Immunocytochemical YB-1 staining in short-term cell culture of endometrial cells (Eo, n = 6; Co, n = 6). The purity of epithelial (A) and stromal (B) cell cultures from endometrial biopsies were confirmed by using cytokeratin staining (CK18; magnification, ×400). (C) No significant YB-1 staining was detected in epithelial cells from Co samples (magnification, ×400). (D) Negative control of YB-1 staining in Co epithelial cells (magnification, ×200). (E) Strong YB-1 staining could be observed in endometrial epithelial cells from women with Stage I Eo (magnification, ×200). (F) No YB-1 staining was detected in endometriotic epithelial cells of negative controls (magnification, ×200). (G and H) An absence of YB-1 staining was also observed in endometrial stromal cells from Eo patients and in the negative control, respectively (magnification, ×200).
indicated its mitogenic, angiogenic, migrating and invading nature (Banu et al., 2008). Moreover, Banu et al. (2009) have demonstrated that the transplantation of human immortalized Eo epithelial Z12 cells (mixed with the immortalized endometriotic stromal B22 cell line) into the peritoneal cavity of the recipient nude mice was able to attach, proliferate, invade, reorganize and establish peritoneal Eo. Based on these data, the Z12 cell line was selected for our experiments as a good model to study the molecular functions of YB-1 in Eo.

Altogether, we believe our findings underline the theory that the transcription factor YB-1 has an impact on the development and progression of Eo. Furthermore, YB-1 shows high potential for different therapeutic approaches, such as specific knockdown, resulting in

Figure 5 Effect of YB-1 knockdown in an epithelial Eo cell line. (A) Decreasing YB-1 protein expression was detected in YB-1 siRNA transfected Z12 cell lines (YB-1 siRNA) by western blotting at 1–4 days after transfection. Lowest YB-1 levels were consistently observed at 3 and 4 days after YB-1 siRNA transfection. No significant alteration was verified in non-targeting siRNA-transfected cells (NT siRNA). (B) Cell proliferation was monitored after 4 days of siRNA transfection using the MTT assay. Cell growth was significantly reduced in siRNA-transfected Z12 cell line in comparison with NT siRNA and mock (transfection media only; Mock) cells as well as untransfected cells (Untransf; \( \cdot P < 0.001 \)). In this assay, cell death control siRNA-transfected cells (Cell death) were also used as Co. (C) Apoptosis was investigated by DNA fragmentation with a nucleosomal fragment detection enzyme-linked immunoassay. YB-1 knockdown promoted significant spontaneous and TNFα-induced apoptosis in YB-1 siRNA cells (\( \cdot \cdot \cdot P < 0.05 \)). (D) Low expression of YB-1 was associated with higher CCL5/RANTES levels (detected by ELISA-based assay) in TNFα-stimulated cell cultures treated by YB-1 siRNA in comparison with untransfected cells (\( \cdot \cdot \cdot \cdot P < 0.05 \)). (E) The effect of YB-1 knockdown was also observed on cell invasion in vitro. The invasive phenotype of YB-1 siRNA cells was detected using a Matrigel assay. Each experiment was performed with a minimum of three times in triplicate.
decreased proliferation and growth of ectopic endometrial cells in affected tissues.

**Authors’ roles**

B.R., K.D., D.H. and A.A. contributed to the study design. J.K. and C.G.T.S. conducted the data analyses and statistics. All authors contributed to the interpretation of the data and were involved in the critical revision and final approval of the paper.

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