Syndecan-1 knockdown in endometrial epithelial cells alters their apoptotic protein profile and enhances the inducibility of apoptosis

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Abstract: Endometrial epithelial cells are known to undergo apoptosis during trophoblast invasion. We postulate that the cell surface molecule Syndecan-1 which is expressed on endometrial cells and syncytiotrophoblast is important for implantation in general and especially for induction of maternal cell apoptosis during trophoblast invasion because Syndecan-1’s influence on apoptotic susceptibility of cancer cells is already described in the literature. Using the human endometrial epithelial cell line RL95-2, a new stable cell line with Syndecan-1 knockdown was generated. Via antibody array analysis, a significant decrease in the expression of anti-apoptotic proteins like inhibitors of apoptosis, Clusterin, heme oxygenase (HO-2), heat shock protein (HSP)27 and -70 and Survivin due to the Syndecan-1 knockdown was discovered. Correspondingly, active Caspase-3 as an indicator for apoptosis was increased more severely in these cells compared with unmodified RL95-2 after treatment with implantation-related stimuli, which are the cytokines interleukin-1β, interferon-γ, tumor necrosis factor-α and transforming growth factor-β1 and an anti-Fas antibody. Furthermore, a treatment with a combination of all factors caused a higher Caspase-3 induction compared with each single treatment. These results demonstrate that Syndecan-1 is involved in the control of apoptosis in RL95-2 cells and therefore may affect the fine tuning of apoptosis in endometrial epithelium regulating the embryo’s invasion depth as a crucial step for regular implantation followed by successful pregnancy.

Keywords: embryo / Fas / IAPs / implantation / RL95-2

Introduction

Trophoblast invasion in humans is strictly regulated regarding space and time. After attachment to the maternal endometrial epithelium, the trophoblast disrupts the epithelium and invades the decidua as far as the proximal third of the myometrium (Bischof et al., 2000). A proposed mechanism for epithelial cell destruction is via apoptosis mediated by Fas receptor (Fas) and Fas ligand (Fasl), since the trophoblast expresses Fasl (Uckan et al., 1997) and the corresponding receptor is found on endometrial epithelial cells (Hsu et al., 2008). Co-culture experiments with BeWo trophoblast spheroids and RL95-2 endometrial epithelial cells (EEC) revealed apoptosis at the spheroid-EEC interface (Li et al., 2003) and enhancement of EEC apoptosis and spheroid expansion by an anti-Fas activating antibody (ab) (Hsu et al., 2008). Furthermore, ultrastructural studies of the implantation site in rodents revealed that EECs surrounding the embryo undergo apoptosis and are phagocytosed by the trophectoderm (Schlaak et al., 1985; Parr et al., 1987).

Apoptosis, or programmed cell death, is characterized by fragmentation and enfolding of cell compartments into membrane-covered apoptotic bodies that are removed without any immune response or damage of the surrounding cells (Kerr et al., 1972). It is tightly regulated by a cascade of caspases which are distinguished in initiator caspases, like Caspase-8 and -9 at the beginning of the pathway and following effector caspases, like Caspase-3 mediating the cellular morphological changes (Henson et al., 2001). Apoptosis can be induced via the extrinsic pathway which is initiated by binding of an external ligand to the corresponding death receptor, i.e. Fas, tumor necrosis factor (TNF) receptor (R) or TNF-related apoptosis-inducing ligand receptor (TRAIL R), leading to an activation of Caspase-8 which subsequently initiates the intracellular cascade (Kischkel et al., 1995) and the intrinsic pathway initiated via the permeabilization of mitochondria and release of...
Cytochrome C, activating Caspase-9. Subsequently, both pathways lead to the activation of Caspase-3 and are regulated by several pro- and anti-apoptotic molecules. The inhibitor of apoptosis (IAP) family includes different members like XIAP, cIAP-1, -2 and Survivin which can bind directly to caspases and thereby inactivate them (Deveraux and Reed, 1999). On the opposite side pro-apoptotic molecules like Second mitochondria-derived activator of caspases (SMAC) and High temperature requirement protein A2 (HtrA2) which are released of the mitochondria together with Cytochrome C bind IAPs and attenuate or even prevent their inhibitory effects on apoptosis (van Loo et al., 2002). The interaction of pro- and anti-apoptotic molecules is well balanced in the cell but a shift to pro-apoptotic proteins leads to the induction of apoptosis and consequently the cell death.

Syndecan-1 (Sdc-1) is a member of a family of cell surface heparan sulfate (HS) proteoglycans. At the cell surface, Sdc’s act as co-receptors and storage molecules, affecting the stability, conformation or oligomerization state of ligands and receptors or increasing ligand concentration through their HS chains. In contrast, proteolytical cleavage of intact Sdc ectodomains with all their HS chains leads to soluble effectors through their HS chains. In contrast, proteolytical cleavage of intact Sdc ectodomains with all their HS chains leads to soluble effectors in the extracellular milieu (Gotte and Echtnermeyer, 2003; Hayashida et al., 2006). In general, Sdc’s possess high influence on physiological and pathological processes by regulating cell – cell interactions, migration, concentration, development and tumorigenesis (Bernfield et al., 1999). Sdc-1 mRNA and protein were found in endometrium of normal cycling healthy women with a main localization in glandular epithelium and a significant increase in secretory phase compared with proliferative cycling healthy women with a main localization in glandular epithelium and pathological processes by regulating cell–cell interactions, migration, concentration, development and tumorigenesis (Bernfield et al., 1999).

Recent publications about the influence of Sdc-1 on apoptosis revealed aJanus-faced attitude, since in some cancer types a low Sdc-1 expression was correlated with high malignancy (Matsumoto et al., 1997; Pulkkinen et al., 1997; Anttonen et al., 1999, 2001; Mikami et al., 2001; Numaa et al., 2002), whereas in other cancer types the contrary was observed (Barbareschi et al., 2003; Ito et al., 2003; Davies et al., 2004; Chen and Ou, 2006). In endometrial cancer Sdc-1 expression was significantly up-regulated (Choi et al., 2007). Furthermore, overexpression of Sdc-1 promoted endometrial carcinoma cell proliferation and invasion which was correlated with activation and nuclear translocation of nuclear factor (NF)κB (Oh et al., 2009). Correspondingly, silencing of Sdc-1 caused apoptotic cell death of endometrial carcinoma cells and Sdc-1kd mediated apoptosis was correlated with a decrease in the activation of MAPK (Mitogen-activated protein kinase) Erk and Akt (Choi et al., 2007). An influence of Sdc-1 on the Akt-pathway was also described for prostate cancer cells (Hu et al., 2010). Another study about the role of Sdc’s in death receptor mediated cell death of multiple myeloma cells described that Sdc-1 kd enhanced the sensitivity to TRAIL-induced apoptosis, while Sdc-2 and -4 kd increased the sensitivity to FasL (Wu et al., 2012). In spite of the contrary literature an influence of Sdc-1 kd in EEC apoptosis by modulation of MAPK, Akt and NFκB or death receptor signaling is conceivable although up to date the knowledge about the exact mechanism underlying the influence of Sdc-1 on apoptosis is very limited.

Therefore, the purpose of this study was to generate a stable kd of Sdc-1 in EECs to further clarify its physiological role in human implantation with regard to regulation of apoptosis during this process. To gain this information, a detailed investigation of pro- and anti-apoptotic proteins involved in regulation of apoptosis mediated by Sdc-1 was performed as well as the induction of apoptosis demonstrated by the activation of Caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage due to embryonic stimuli.

### Materials and Methods

#### Human cell lines

Human endometrial epithelial carcinoma cell line RL95-2 was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA; CRL 1671; Way et al., 1983). Cells were maintained at 5% CO2 and 37°C in DMEM:F12 with 15 mM HEPES (Biowest, Nuaillé, France), supplemented with 5% (v/v) charcoal-stripped fetal calf serum (FCS) (or Tet-free fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) starting with Tet-induction), 1 × penicillin/streptomycin, 1 × sodium pyruvate, 2 mM l-glutamine (all Biowest), 20 μg/ml insulin and 5 μg/ml holo-transferrin (both Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 5% CO2 and 37°C.

#### Transfection of pcDNA™/TR

The T-REx™ System (Invitrogen, Karlsruhe, Germany) was chosen to generate a stable, tetracycline (Tet) inducible Sdc-1 kd in RL95-2 cells. First, the regulatory vector pcDNA™/TR, coding for the Tet-repressor (R), was transfected, to generate a stable TetR expression host cell line, using a Nucleofector™ I (Lonza Cologne GmbH, Cologne, Germany) and the transfection reagent Nanofectin (GE Healthcare, Freiburg, Germany), respectively. For Nucleofection®, 5 × 10⁵ cells were transfected with 3 μg vector DNA in buffer V (Lonza Cologne GmbH) using the programs: T-13, T-20, W-1 and afterward seeded on a 35 mm dish. For chemical transfection with Nanofectin, 4 × 10⁵ cells were mixed with 3 μg DNA and 9.6 μl reagent, seeded on a 35 mm dish and transfected for 24 h according to manufacturer’s instruction. The selection with 3.5 μg/ml Blastcidin S (Invivogen, San Diego, CA, USA), as tested before via a killing curve started 48 h after Nucleofection® or 24 h after Nanofectin transfection.

#### Real-time PCR for TetR

Real-time PCR analysis with specific primers for the pcDNA6/TR sequence was performed. Primers were: for 5′-GCC AGG ATG ACA GCC TTTAG-3′ and rev 5′-GGT GTT GCC TCT TCT CCT TTT G-3′ (annealing temperature 56°C). For untransfected RL95-2 control cells which did not express TetR products, the ΔCt with normalization to β–Actin was calculated (Livak and Schmittgen, 2001) and further compared with successfully pcDNA/TR transfected endometrial stroma cells generated in our laboratory (Baston-Büst et al., 2010). Two clones with similar ΔCt were chosen for further transfection.

#### Design, cloning, sequencing and transfection of short hairpin (sh) RNA

Using Invitrogen’s® RNAi designer, following shRNA oligonucleotides for the Sdc-1 kd were designed: top strand 5′-CAG AGG AGT TCT TGC TTG CCA AGA TAT CGG AGG A TA TCT TGC AAA GCA CCT GC′-3′, bottom strand 5′-AAA AGC AGG TGC TTT GCA GA A TAT CTT GGA ATA TCT TGC AAA GCA CCT GC-3′; Sdc-1 database sequence AJ551176.1, Location: 393 GCA GGT GTT TTG CAA GAT TTC 414. Sequences of shRNA covering Sdc-1 messenger RNA are shown in bold. The first four bases of the shRNA oligonucleotides are required for direct cloning in the vector pENTR™/H1/TO; the cursive printed bases are required for the loop sequence. For ligation, double-stranded oligonucleotides and pENTR™/H1/TO vector were mixed in a molar ratio of 50:1 and ligation was performed according to the manufacturer’s protocol and transformed...
into chemically competent Escherichia coli Top10. Selection of transformands was performed with 50 μg/ml Kanamycin (Invitrogen). Ten positive clones were cultured and analyzed after plasmid DNA isolation (GeneJet™ Plasmid Miniprep Kit, Fermentas, St. Leon-Rot, Germany) by sequencing with H1 forward and M13 reverse primers (Invitrogen) at the biomedical research center (BMFZ) of the Heinrich-Heine University (Düsseldorf, Germany). Clones identified with in frame inserted shRNAs for Sdc-1 were transfected into the TetR expressing host cell line with Nanofectin as described above, followed by selection with 200 μg/ml Zeocin and 3.5 μg/ml Blasticidin S (both Invitrogen). Successfully double-transfected cells contain the TetR protein encoding vector pcDNA™TR/IRE and the vector pENTR™H1/TO encoding the Sdc-1 shRNA downstream of the H1/TO pol III promoter which contains Tet-Operator 2 (TetO2) binding sites for Tet-regulated expression. Without Tet the TetR binds the TetO2 sites and blocks the shRNA transcription. After addition, Tet binds the TetR leading to conformation changes and subsequent to release from the TetO2 sites which then transcribe the Sdc-1 shRNA.

**Tet-induction and analysis of Sdc-1 kd in double-transfected RL95-2 on mRNA and protein level**

Successfully selected EEC clones were treated with 1 μg/ml Tet (Invitrogen) for 24 h according to the manufacturer’s protocol to induce the kd of Sdc-1. mRNA was analyzed by real-time PCR using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Primers for Sdc-1 were for 5′-GGGACATAACGGCTG TAGTGG-3′ and rev 5′-AGCTGCGCTCCTCGTCCTGTC-3′ (annealing temperature 53°C). Two clones with the lowest expression of Sdc-1 mRNA after Tet-induction were investigated for Sdc-1 protein expression in membrane protein fraction via dot immunoblotting. Membrane protein fraction was isolated via Subcellular Protein Fractionation Kit (Pierce Biotechnology, Rockford, IL, USA). For dot blotting, a stack of five dry and five tank fraction was isolated via Subcellular Protein Fractionation Kit (Pierce Biotechnology, Rockford, IL, USA) for 3.5, 7 and 14 h; with a combination of 10 ng/ml IL-1β, 10 ng/ml IFN-γ, 5 ng/ml TNF-α, 0.5 ng/ml TGF-β1 for 24 h and additionally with 5 μg/ml anti-human CD95 (Fas) ab clone EOS9.1 (all Biolegend) for another 7 h. Protein lysates were prepared with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer’s protocol. Thirty-microgram protein was separated on a PVDF membrane (Merck Millipore). The membrane was blocked with 5% non-fat milk and incubated with antibodies against PARP (9542, Cell Signaling Technology) and β-Actin (ab6276, Abcam) at 4°C overnight.

**Western blot analysis**

RL95-2 and Tet-induced RLsdc1kd were treated with 10 ng/ml IL-1β, 10 ng/ml IFN-γ, 5 ng/ml TNF-α, 0.5 ng/ml TGF-β1 for 24 h and additionally with 5 μg/ml anti-human CD95 (Fas) ab clone EOS9.1 (all Biolegend) for another 7 h. Protein lysates were prepared with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer’s protocol. Thirty-microgram protein was separated on a PVDF membrane (Merck Millipore). The membrane was blocked with 5% non-fat milk and incubated with antibodies against PARP (9542, Cell Signaling Technology) and β-Actin (ab6276, Abcam) at 4°C overnight.

**Screening of apoptosis-related proteins**

The expression of 35 different apoptosis-related proteins of total protein lysate was analyzed via the Proteome Profiler™ Human Apoptosis Array Kit (R&D Systems). Briefly, RL95-2 and Tet-induced (1 μg/ml, 24 h) RLsdc1kd cells were first not treated to analyze the initial expression and afterwards treated with 10 ng/ml IL-1β, 10 ng/ml IFN-γ, 5 ng/ml TNF-α, 0.5 ng/ml TGF-β1 for 24 h and additionally with 5 μg/ml anti-human CD95 (Fas) ab clone EOS9.1 (all Biolegend) for another 7 h. Cell extract preparation for the array with 400 μg protein was performed according to the manufacturer’s protocol. Dot blots were photographed and analyzed with an Alpha Imager camera (Biozym Scientific GmbH, Hessisch Oldendorf, Germany).

**Statistical analysis**

Two groups were analyzed applying unpaired two-tailed Mann-Whitney U-test and ANOVAs with Bonferroni post hoc tests for multiple comparisons by using SPSS 21 (SPSS, Chicago, IL, USA). Values of P < 0.05 were considered statistically significant.

**Results**

**Sdc-1 kd in RL95-2**

First, the regulatory vector pcDNA™/TR, encoding the TetR, was transfected, to generate a stable TetR expression host cell line. Due to the epithelioid character of the cells, RL95-2 stained unspecifically with common anti-TetR ab (MoBiTec, Göttingen, Germany; data not shown). Therefore, real-time PCR with specific primers for the pcDNA6/TR sequence was used to compare expression levels of transfected cells versus stroma cells successfully generated and validated in our laboratory before (Baston-Büst et al., 2010). As expected, untransfected RL95-2 did not express the vector. In contrast, three different RL95-2 TetR clones showed pcDNA6/TR expression comparable to the stromal control cells (Fig. 1A) and were subsequently chosen for further transfection with the Sdc-1 shRNA expression vector pENTR™H1/TO. Successfully double-transfected clones were induced with Tet and showed a decrease of Sdc-1 mRNA of >80% (Fig. 1B). This result on mRNA level was further validated on protein level applying a dot immunoblotting analysis showing a weak Sdc-1 expression on the cell membrane of the Sdc-1 kd cells compared with non-induced control cells with normal Sdc-1 expression (Fig. 1C).
Expression of apoptosis-related proteins

The initial expression of 35 different apoptosis-related proteins in whole protein lysates of RL95-2 compared with Tet-induced RLSdc1kd without any further treatment and induction of apoptosis was investigated with the Proteome Profiler Human Apoptosis Array Kit. The expression of the intrinsic, pro-apoptotic proteins Bad, Bax, HtrA2 and SMAC was not altered by Sdc1 kd, whereas Cytochrom C was increased in RL95-2. Within the group of extrinsic death receptors TNF R1 was increased in normal RL95-2 while the receptors TRAIL, Fas and the receptor adaptor molecule FADD (Fas-associated protein with death domain) did not vary (Fig. 2A). In contrast to the rather small alteration within the group of pro-apoptotic proteins there were a lot of changes in the group with known anti-apoptotic abilities like cellular inhibitor of apoptosis (cIAP)-1 and -2, X-linked inhibitor of apoptosis (XIAP), Survivin, Clusterin, heme oxygenase (HO-2) and HSP27 and -70 which were statistically significant decreased in RLSdc1kd compared with RL95-2. The anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-x were expressed comparably under both conditions (Fig. 2B). The same was found for cell cycle checkpoint and control proteins p21, p27, pRad17 and different phosphorylation forms of p53 (Fig. 2C).

Induction of apoptosis by embryonic stimuli

RL95-2 and Tet-induced RLSdc1kd were treated with an anti-Fas ab that binds to the receptor and induces apoptosis similar to FasL which is physiologically expressed by the trophoblast. After different time points (3.5, 7, 14 and 24 h) active Caspase-3 as a marker for apoptosis induction was measured. Additional cells were pre-treated with a combination of IL-1β, IFN-γ, TNF-α and TGF-β1 (IITT) for 24 h before anti-Fas ab was applied for another 7 h. Treatment with anti-Fas ab for 7 h increased the active Caspase-3 4.8 fold in both RL95-2 and RLSdc1kd compared with untreated controls. Incubation with IITT alone for 24 h did not affect the Caspase-3 activation. However, pretreatment with IITT before 7 h anti-Fas ab incubation enhanced the active Caspase-3 more than anti-Fas ab incubation alone (9.6 fold in RL95-2 and 9.9 fold in RLSdc1kd). Caspase-3 induction was significantly higher in RLSdc1kd compared with RL95-2 after treatment with the combination of IITT and anti-Fas ab and anti-Fas ab alone for 7 and 14 h (Fig. 3A). Incubation with IITT and anti-Fas ab led to the highest activation of Caspase-3 which was statistically significant increased compared with untreated controls and the other single treatments. Consequently, this incubation condition was chosen for all further experiments.

Additionally, cleavage of PARP as the main downstream target of active Caspase-3 was detected after incubation with IITT and anti-Fas ab (Fig. 3B). Uncleaved PARP is involved in DNA repair but cleavage facilitates cellular depletion and serves as a marker for cells undergoing irreversible apoptosis (Oliver et al., 1998).

To study the activation of Caspase-8 and -9 as representatives for the extrinsic and intrinsic apoptosis pathway incubation with IITT for 24 h and additionally anti-Fas ab for 7 h was chosen. Extrinsic pathway mediating Caspase-8 was significantly increased 2.2 fold (RL95-2) and 2.4 fold (RLSdc1kd) while intrinsic pathway mediating Caspase-9 was significantly induced 1.7 fold (RL95-2) and 2.0 fold (RLSdc1kd) after treatment. No statistically significant differences were observed between the normal RL95-2 and those with Sdc-1 kd (Fig. 3C).
Expression of apoptosis-related proteins after induction of apoptosis via embryonic stimuli

Treatment of RL95-2 and RLSdc1kd with IITT and anti-Fas ab caused the highest activation of Caspase-3 and cleavage of PARP. Therefore, this condition was chosen to analyze the apoptotic protein expression after incubation with embryonic stimuli to mimic the situation of embryo implantation. After induction of apoptosis, the expression of most investigated (pro- and anti-apoptotic) proteins was significantly up-regulated compared with the initial expression (Table I). Specifically, the anti-apoptotic protein levels in RLSdc1kd did not vary from the RL95-2 without Sdc-1 kd anymore (Fig. 4B) as it was observed for the

Figure 2: Apoptosis Ab Array of untreated control RL95-2 versus RL Sdc1kd. Shown are the pixel densities of different apoptosis-related proteins divided into groups according to their cellular function. (A) Pro-apoptotic intrinsic proteins, extrinsic death receptors and adapter molecules. (B) Proteins with anti-apoptotic impact. (C) Cell cycle checkpoint and control factors. Data represent means ± SEM of four independent experiments, *p < 0.05 RL95-2 versus RLSdc1kd.
untreated RLSdc1kd in the first set of experiments. Instead, the expression of the receptors Fas and TRAIL 2 was significantly up-regulated in RLSdc1kd after treatment with IITT and anti-Fas ab (Fig. 4A).

Table I displays an overview of statistically significant differences in the expression of proteins tested with the ab array in untreated cells as shown in Fig. 2 compared with those after induction of apoptosis as shown in Fig. 4 but this time within each cell group (RL95-2 or RLSdc1kd). Treatment of RL95-2 with IITT and anti-Fas ab elevated the expression of pro-apoptotic HtrA2 and the death receptors Fas, TRAIL 1/2, as well as the expression of the receptor co-molecule FADD. Anti-apoptotic XIAP, Clusterin, HSP60 and -70 increased likewise, while the phosphorylation forms S15 and S392 of the cell cycle control factor p53 decreased after treatment. In IITT and anti-Fas ab treated RLSdc1kd cells all tested pro- and anti-apoptotic proteins except SMAC, Bcl-2, Bcl-x increased compared with untreated controls, within the group of cell cycle control factors p21 was elevated as well after treatment and induction of apoptosis.

Discussion

In the present study, a new EEC line with a stable kd for Sdc-1 named RLSdc1kd was generated to investigate Sdc-1’s properties regarding apoptosis in EECs. Additionally, Sdc-1’s specific role in human implantation-related apoptosis initiated by embryonic stimuli was examined. An influence of Sdc-1 on the apoptotic susceptibility of different cancer cells of the female reproductive tract is known and described in the current literature (Numa et al., 2002; Davies et al., 2004; Choi et al., 2007; Oh et al., 2009). In this context, a Janus-faced attitude of Sdc-1 concerning the regulation of apoptosis in connection with the pathogenesis of tumor cells was revealed. Although embryo implantation is a benign process...
Figure 4  Apoptosis Ab Array of RL95-2 and RLSdc1kd after treatment with a combination of 10 ng/ml IL-1β, 10 ng/ml IFN-γ, 5 ng/ml TNF-α, 0.5 ng/ml TGF-β1 (ITIT) for 24 h and additionally with 5 μg/ml anti-Fas ab for another 7 h. Shown are pixel densities of different apoptosis-related proteins divided into groups according to their cellular function. (A) Pro-apoptotic intrinsic proteins and extrinsic death receptors and adapter molecules. (B) Proteins with anti-apoptotic impact. (C) Cell cycle checkpoint and control factors. Data represent means ± SEM of five independent experiments, *P < 0.05 RL95-2 versus RLSdc1kd.
process, similarities to cancer invasion are apparent. It is known that Sdc-1 mRNA is expressed in endometrium of normal cycling healthy women with a significant increase in secretory phase epithelial endometrium compared with proliferative phase. Accordingly, Sdc-1 protein expression increased in the secretory phase endometrium with a main localization in glandular epithelium (Germeyer et al., 2007). This indicates that an increase of Sdc-1 expression is strongly associated with the place and time point of implantation which suggests a potential role of Sdc-1 in regulating trophoblast invasion and implantation possibly by influencing EEC apoptosis. To clarify the regulatory role of Sdc-1 in EEC death, the protein expression pattern of different apoptosis-related factors was examined. Without external induction of apoptosis the expression of several different proteins with known anti-apoptotic abilities like Bcl-x, Bcl-2, cIAP-1 and -2, XIAP, Survivin, Clusterin, HO-2 and HSP27 and -70 as well as the receptor TNF R1 was significantly decreased in RLSdc1kd cells compared with regular RL95-2 controls. CIAP-1 and -2, XIAP and Survivin are members of the IAP family, which serve as suppressors of apoptosis induced by e.g. TNF-α and FasL, by direct caspase inhibition (primarily Caspase-3 and -7) and modulation of the transcription factor NFκB (LaCasse et al., 1998). TNF R1 can trigger both the pro-apoptotic and anti-apoptotic response in cells. Apoptosis can be initiated via Caspase-8 activation as it was described before for death receptors in general. But instead of Caspase-8 the IkB Kinase (IKK) can also be activated after recruitment to TNF R1, which leads to an inactivation of the inhibitor IkB and correspondingly the activation of NFκB signaling, which mediates anti-apoptotic effects in the cell (Chen and Goeddel, 2002). Furthermore, XIAP is described to mediate first trimester trophoblast resistance to Fas-mediated apoptosis in spite of expressing both Fas and FasL (Straszewski-Chavez et al., 2004). Characterizing the biological function of Clusterin is difficult because of its tendency to interact with a broad range of molecules and its up-regulated expression even leading to different partially contradictory cellular processes (Jones and Jomary, 2002). Additionally, Clusterin is described to have protective, anti-apoptotic and chaperone-like abilities (Trougakos and Gonos, 2002; Wang et al., 2012).

It is known that HSP27 and -70 are increased in the endometrium after ovulation and in the early secretory phase, as the critical period of endometrial receptivity for an implanting embryo. This expression is correlated with a possible protection of endometrial cells from adverse side-effects of cytokine release, because it is known that HSP70 protects from cytotoxic damage by TNF-α and inhibits apoptosis (Neuer et al., 2000). Another study describes HSP27 as well as -70 as anti-apoptotic proteins that directly interact with several apoptotic effectors like SMAC, AIF (apoptosis-inducing factor) or FADD (Garrido et al., 2006). No inter molecular interaction of Sdc-1 on anti-apoptotic proteins which were shown to be decreased in this study is described in the current literature so far. A possible hint though might be that Sdc-1 kdk induced apoptosis in endometrial cancer cells correlated with a decreased activation of Akt (Choi et al., 2007). Akt as the main mediator of PI3K-signaling is known to modulate apoptosis- and survival-related proteins. For instance it inactivates pro-apoptotic Bad and activates anti-apoptotic XIAP, Survivin and the NFκB-pathway, which subsequently induces the expression of IAPs (Chang et al., 2003; Li et al., 2012). Based upon this hypothesis the down-regulation of anti-apoptotic proteins in the current investigation could be mediated via inhibition of Akt signaling in the Sdc-1 kdk cells. Furthermore, Sdc-1 overexpression in endometrial cancer cells promotes cell proliferation via NFκB (Oh et al., 2009) which supports the hypothesis of an influence of Sdc-1 on the Akt signaling pathway that subsequently modulates apoptosis.

The weaker expression of anti-apoptotic proteins in RLSdc1kd is mirrored in higher apoptotic susceptibility after treatment with implantation-related factors as displayed by higher active Caspase-3. Implantation is tightly regulated by a multitude of cytokines secreted by the trophoblast and the maternal endometrium. It is also described that EEC apoptosis is initiated by the interaction of FasL-bearing trophoblast and maternal cells with the corresponding receptor Fas (Galán et al., 2000). To simulate the in vivo situation, the cytokines IL-1β, IFN-γ, TNF-α and TGF-β1 (IITT) as well as an anti-Fas ab were chosen for treatment of RL95-2 and RLSdc1kd. High levels of IL-1β are secreted by human embryos in vitro (Sheth et al., 1991; Krüssel et al., 1998) while the receptor is highly expressed on the maternal epithelium during the peri-implantation period (Simón et al., 1994). IFN-γ is expressed by early trophoblast

<table>
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<tr>
<th>Table I</th>
<th>Summary of statistically significant differences of the initial apoptotic protein expression in untreated controls (Fig. 2) versus the expression after induction of apoptosis by treatment with IITT and anti-Fas ab (Fig. 4).</th>
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<td>Control versus treated</td>
<td>RL95-2</td>
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*P < 0.05.
and uterine NK cells whose number rapidly expands in early pregnancy (Murphy et al., 2009). TNF-α and the corresponding receptors are found in endometrium, placenta and the fetus during pregnancy (Zollner et al., 2012). TGF-β1 is expressed in endometrial stromal and epithelial cells (Kauma et al., 1990), in the syncytiotrophoblastic layer (Dungy et al., 1991) and in the first trimester chorionic villi of normal placenta (Xuan et al., 2007). Regarding Sdc-1 it is known that it acts in general as a co-receptor for cytokines and the direct binding of HS is described for IFN-γ, TNF-α and TGF-β1 already. Therefore, an influence of Sdc-1 as an HS chain carrier in endometrial epithelial cells is possible (Göttle, 2003). Taken together the cytokines chosen for the experiments are known to be secreted by the trophoblast during implantation and therefore represent a reduced but suitable substitute for the presence and secretome of an implanting embryo in absence of better possibilities since the German Embryo Protection Act prohibits the use of human embryos for research purposes. Hence it needs to be kept in mind that the results of the current experiments could possibly vary if an embryo would be used instead of some of its secreted products.

After treatment of RL95-2 and RLSdc1kd with anti-Fas ab active Caspase-3 was significantly increased after 7 h compared with untreated controls. Additionally active Caspase-3 was significantly elevated in RLSdc1kd compared with RL95-2. Incubation with ITITT alone did not alter the activation of Caspase-3 compared with control, but appears to sensitize the cells for Fas-mediated apoptosis, because incubation with anti-Fas ab after pretreatment with the cytokines increased active Caspase-3 compared with single anti-Fas ab incubation. Within these incubation conditions Caspase-3 was more activated in RLSdc1kd compared with RL95-2. It was published before that TGF-β1 and IL-1β pretreatment enhances Fas-mediated apoptosis in EECs (Tanaka and Umesaki, 2000). Corresponding to the high amount of active Caspase-3 after incubation with the cytokines and anti-Fas ab cleaved PARP as the main downstream target of active Caspase-3 and a marker for cells undergoing apoptosis could be observed in the present study. The induction of apoptosis in both cell types after incubation with embryo-related stimuli indicates a possible role for maternal apoptosis during embryo invasion to establish the necessary space for proper implantation. Since active Caspase-3 is significantly decreased in normal RL95-2, Sdc-1 is supposed to extinguate the apoptotic signal and therefore may regulate the invasion depth which is a crucial criterion for a successful pregnancy.

Activation of Caspase-8 and -9 was further investigated to find out which apoptotic pathway was initiated and led to the activation of Caspase-3. Interestingly not only the extrinsic pathway via death receptor associated Caspase-8 was switched on as expected after anti-Fas ab treatment but also intrinsic Caspase-9 was activated compared with untreated control, which might be a secondary effect of mitochondrial damage initiated by the protein BID (BH3 interacting domain death agonist) after cleavage by Caspase-8 as already described (Li et al., 1998). In contrast to the direct influence of Sdc-1 on Caspase-3 observed in this study, indicating an influence of the kd on molecules regulating activation of Caspase-3, no statistical significant difference could be seen for an involvement of Sdc-1 in Caspase-8 and -9 activation.

The higher apoptotic susceptibility of RLSdc1kd, mirrored by higher active Caspase-3, can also be correlated with an influence of Sdc-1 on the Akt- and correspondingly NFκB-pathway. As it is already described for endometrial cancer cells Sdc-1 kd decreases anti-apoptotic Akt and induces apoptosis (Choi et al., 2007) while Sdc-1 overexpression prevents apoptosis via NFκB as a direct activated target of Akt (Oh et al., 2009).

Treatment with the combination of cytokines and anti-Fas ab as an imitation of the embryo’s secretion products mimicking the embryomatratal crosstalk led to the highest activation of Caspase-3 compared with untreated controls and furthermore a significant enhancement of active Caspase-3 in the Sdc-1 kd cells RLSdc1kd compared with regular RL95-2. Therefore, these incubation conditions were chosen to study the pro- and anti-apoptotic protein expression pattern again to investigate the changes due to the embryo’s signal. After induction of apoptosis via ITITT and anti-Fas ab most of the pro- and anti-apoptotic proteins were significantly increased in both cell lines RL95-2 and RLSdc1kd. In particular the pro-apoptotic HtrA2, the receptors TRAIL R1/2 and Fas with the corresponding death receptor adaptor molecule FADD were increased. It was published before that TGF-β1 and IL-1β do not effect the proliferation of the EEC line HHUA, but pretreatment enhances Fas-mediated apoptosis while flow cytometric analysis shows that Fas expression is not influenced by cytokines. The authors conclude that cytokines enhance the apoptotic susceptibility and postreceptor apoptotic signals (Tanaka and Umesaki, 2000). In the present study a higher Fas-mediated apoptotic susceptibility after cytokine treatment could be observed as well but in contrast to the findings of Tanaka and Umesaki (2000) it is correlated with an increased death receptor expression. Furthermore, anti-apoptotic XIAP, Clusterin and HSP60 and −70 were up-regulated. Additionally, in RLSdc1kd cells the anti-apoptotic proteins cIAP1/2, Survivin and HO-1/2 were significantly elevated after induction of apoptosis with the embryonic stimuli. This increased expression did not alter anymore from RL95-2 with normal Sdc-1 as observed before. In contrast, the death receptors Fas and TRAIL R1/2 were significantly increased in RLSdc1kd compared with RL95-2, which might result in a higher responsiveness to induction of apoptosis by the corresponding ligands. These observations demonstrate the simultaneous regulation of pro- and anti-apoptotic proteins reflecting the complexity of apoptotic signaling cascades insofar as increase of pro-apoptotic proteins during induction of apoptosis implicates an induction of opponent anti-apoptotic proteins at least in part.

Furthermore, it is already described that TGF-β1 up-regulates XIAP (Caja et al., 2011) and TNF-α as well as IL-1β can induce IAPs via NFκB (Karim and Lin, 2002). Thus the increase of apoptotic proteins during apoptosis may also be regulated by ITITT treatment independent of Sdc-1. But even with this increase of anti-apoptotic proteins via embryonic stimuli cells with Sdc-1 kd demonstrated a higher active Caspase-3 expression reflecting Sdc-1’s role in apoptosis.

In accordance with the findings that death receptors Fas and TRAIL R1/2 are increased in RLSdc1kd during apoptosis an influence of Sdc kd on death receptor-induced apoptosis of multiple myeloma cells was recently described in the literature. Sdc-1 kd sensitized the cells to TRAIL-mediated apoptosis, while kd of Sdc-2 and -4 increased the apoptotic susceptibility to FasL (Wu et al., 2012). Therefore, TRAIL-mediated apoptosis seems to be an interesting target for further studies in endometrial epithelium since elevated levels of TRAIL R1/2 and Fas were observed after induction of apoptosis herein. These results reveal the complex and versatile protein interactions in the signaling pathway of apoptosis which regulate the momentous process of cell death.

At this point it needs to be addressed that the experiments in the current study were performed using the RL95-2 cell line instead of primary cells since the introduction of a stable Sdc-1 kd is not feasible.
in primary epithelial cells. Although this cell line is a well described and a popular model for receptive endometrial epithelium, differences to primary cells may appear.

In summary, a stable Sdc-1 kd cell line was generated which facilitates investigations of EECs with regard to the physiological role of Sdc-1 in the process of human embryo implantation \textit{in vitro}. As a co-receptor and storage molecule for implantation-related cytokines Sdc-1 controls binding of these ligands and following signaling cascades. The data show that Sdc-1 kd cells show a higher apoptotic susceptibility. This is displayed by a significant higher active Caspase-3 initially mediated by lower expression of anti-apoptotic proteins before induction of apoptosis and amplified by a higher expression of death receptors upon induction of apoptosis. Furthermore, treatment of EECs with the combination of IITT together with anti-Fas ab mimicking the embryonic factors secreted during implantation led to the highest induction of apoptosis in both cell types.

Hence Sdc-1 is an important factor regulating apoptosis of the endometrial epithelial cells RL95-2 \textit{influencing} the expression pattern of pro- and anti-apoptotic proteins before and during apoptosis, thereby mediating the susceptibility of the cells for apoptosis induced by embryonic stimuli and therefore may influence as well the precise adjusted regulation of endometrial epithelial cell apoptosis during implantation \textit{in vivo}. Hypothetical mechanisms being subject to the influence of Sdc-1 on EEC apoptosis as shown in Fig. 5 might be via modulation of extracellular cytokine and death receptor binding to their corresponding ligands and regulating post-receptor signaling pathways like MAPK or PI3K with the mediator Akt and the corresponding NFκB-pathway intracellularly. Another conceivable mechanism of Sdc-1 influencing EEC apoptosis could be extenuating the extrinsic Caspase-8-mediated pathway on the one hand or by direct influence on Akt and NFκB on the other hand.

Even if the exact Sdc-1 influence on interactions of apoptosis induction is not fully understood so far the overall findings of this study indicate that Sdc-1 may be involved in the regulation of maternal cell apoptosis during embryo implantation acting as a modulator of invasion depth as a crucial factor for successful pregnancy. Accordingly, different clinical studies correlate a reduced Sdc-1 expression with idiopathic fetal growth restriction (Chui et al., 2012) and a higher expression of Sdc-1 with a reduced risk of preterm delivery (Schmedt et al., 2012). Since both diseases are correlated with inadequate implantation, these data also suggest that Sdc-1 appears to be an important factor influencing invasion depth probably by regulating maternal endometrial cell apoptosis. This knowledge offers interesting points for investigations regarding clinical pathologies. It is possible that an excessive apoptosis as observed in RLSdc1kd could cause incorrect implantation leading to pregnancy disorders which needs to be further investigated.

**Authors’ roles**

S.J.B. conceived the study, conducted all experiments, prepared the figures and the manuscript. D.M.B.-B. assisted in initiation of experimental design, manuscript drafting and critical discussion. O.A.-A. supported in cloning and transfection and participated in manuscript drafting. J.S.K. participated in manuscript preparing and critical discussion. A.P.H. initiated and supervised the project and participated in manuscript drafting and critical discussion.
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Conflict of interest

All the authors declare that there is no conflict of interest.

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