HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Death before Life?

The role of apoptosis in human embryonic implantation

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Life isn't about waiting for the storm to pass…
It's about learning to dance in the rain.
Vivian Greene

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1. Introduction

Research in the field of reproductive biology mainly investigates the physiology of the reproductive tract, early embryonic development after fertilization and the implantation of the embryo into the maternal tissue as a fundamental requirement for the following successful pregnancy. But until now, the cellular and molecular background of these substantial processes is not fully elucidated. Basic scientific findings support the clinical field of reproductive medicine with improvements regarding the maintenance of reproductive health, but also diagnosis and treatment of reproductive problems, and last but not least in the optimized treatment of infertility by applying assisted reproductive technology (ART). Techniques of ART gained more and more importance in human reproduction in the recent years, considering that nearly 10% of German couples are involuntarily childless. Early implantation-related investigations intensively focused on the embryonic side and due to scientific proceedings and improvements the pregnancy ratio after *in vitro* fertilization (IVF) treatment of infertile couples could be elevated up to 40% per cycle[1]. However, this percentage could not be further improved in the last years. This indicates a potential role for unexplained processes in the endometrium influencing the pregnancy outcome and the necessity of investigations to improve the requirements of the maternal side.

This thesis investigates the early embryo-maternal dialog, deciphering cell behavior on the maternal side as a consequence of embryo contact to gain further insights how the female endometrium is involved in the regulation of proper embryo implantation, finally leading to a successful pregnancy and a “take home” baby.

1.1 The human endometrium

The endometrium is the inner layer of the human (and mammal in general) uterus. The adjacent middle layer is named myometrium and consists mainly of smooth muscle cells. The endometrium is the place of the early conceptus’ nidation, also called implantation. The histological composition of the endometrium includes a single layer of columnar epithelium with luminal and glandular endometrial epithelial cells (EECs), which are secretory or ciliated. The subjacent endometrial stromal cells (ESCs) build a layer of supporting, loose connective tissue with fibroblastic cells, blood vessels (built from vascular smooth muscle and endothelial cells) as well as immune cells and are referred to as the functional layer. The

stromal tissue becomes denser the further it reaches to the myometrium and the cells directly adjacent to the myometrium arrange the basal layer. Furthermore, the endometrium contains tubular glands, which span the epithelial surface through the stroma and sporadically up to the myometrium (Figure 1.1)[2,3].

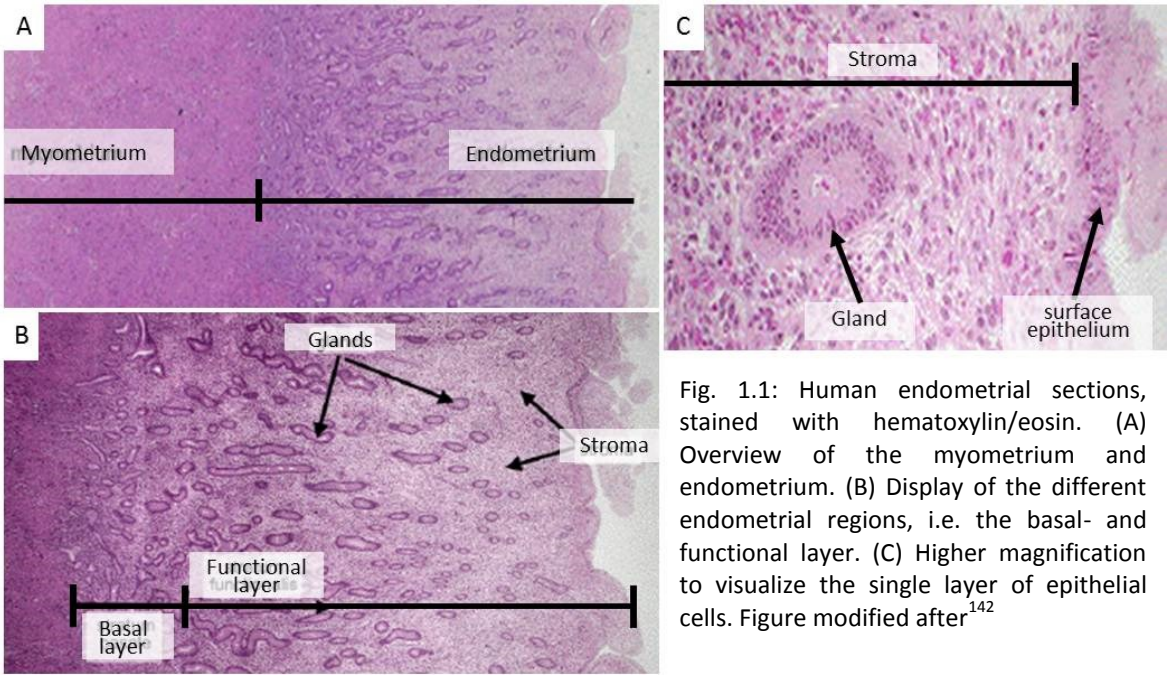

Fig. 1.1: Human endometrial sections, stained with hematoxylin/eosin. (A) Overview of the myometrium and endometrium. (B) Display of the different endometrial regions, i.e. the basal- and functional layer. (C) Higher magnification to visualize the single layer of epithelial cells. Figure modified after[142]

The endometrial functional layer underlies intense morphological and structural changes during the menstrual cycle, determined by the steroid hormones estradiol (E_2) and progesterone (P_4). These changes are elucidated in detail in the following paragraph and can be found schematically illustrated in Figure 1.2.

The cycle starts with the menstruation from day 1–4. If implantation does not occur, circulating E_2 and P_4 decrease and the entire superficial functional endometrial layer is dissociated and discarded with associated bleeding due to the rupture of small blood vessels[4]. The proliferative or follicular phase from day 4–14 of a regular 28 day cycle is initiated via increasing E_2 secreted by the maturing ovarian follicle and is characterized by the formation of a growing endometrial layer due to intense proliferation of endometrial cells, which are associated with the basal layer. Besides proliferation, EEC ciliogenesis can be observed, as well as an elongation of the glands throughout the stroma[3,5].

The proliferative phase ends with an E_2-induced ovulation characterized by a peak production of lutenizing hormone (LH) leading to the release of the mature oocyte into the fallopian tube. The oocyte starts its journey toward the uterus and is either fertilized in the widest section of the fallopian tube, named the ampullary region, or degenerates 12–24

hours after ovulation if fertilization fails to appear. At the time of ovulation the functional layer of the endometrium has reached its maximum size about 7-16mm thickness[6]. In the following secretory or luteal phase from day 14–28 the functional layer of the endometrium differentiates, achieves final maturity, and becomes receptive for the embryo. The dominant hormone of this phase is P_4 secreted by the follicle, which has transformed into a cellular structure (*corpus luteum*) after release of the oocyte[3]. A receptive endometrium is characterized by highly secretory glands, influx of distinct immune cells, and the development of exclusively endometrial-associated, helical sidled arterioles, which grow vertically from the basal layer throughout the functional layer, and designated spiral arteries[7]. Additionally, a biochemical as well as morphological transformation of fibroblast-like ESCs into large, rounded cells, termed decidual cells (dESCs) occurs in a process known as decidualization[8]. This process of ESC remodeling is progressing and continues in a potential pregnancy until the entire endometrium is included and occurs in all species with embryo implantation[9]. However, in human and other menstruating species it arises independently of embryonic signals in every menstrual cycle.

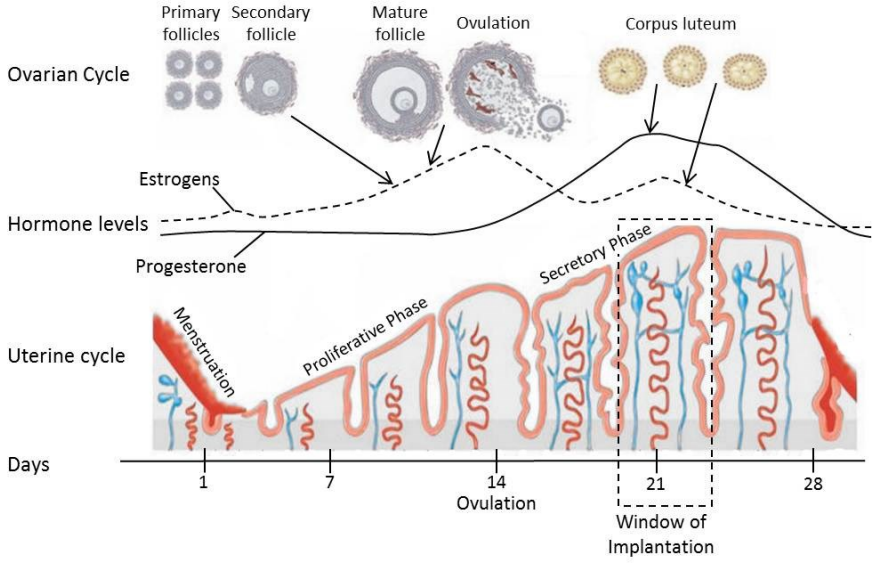

Fig. 1.2: Overview of the human, menstrual cycle. Displayed are the growth/ maturation of a follicle throughout the cycle within the ovary including ovulation and the occurrence of the *corpus luteum*, the ovary derived blood levels of E_2 and P_4 throughout the cycle and changes of the endometrial lining corresponding to the hormonal changes from top to down.

The period in which the endometrium reaches the maximum receptivity for an embryo is between the 20th and 23rd day of the cycle, takes approximately 4 days and is denoted by the "window of implantation"[10]. In the case of a pregnancy, constant increasing P_4 facilitates the decidual maintenance, whereas if no fertilization of the oocyte and subsequent implantation occurs, the *corpus luteum* atrophies causing a P_4 withdrawal, which correspondingly induces menstruation and the beginning of a new menstrual cycle.

1.2 Embryo implantation

Embryo implantation into a receptive endometrium is the pivotal process influencing the success of a following pregnancy until birth tremendously, because the precise extent of invasion as a crucial factor is limited and highly specific[11]. The importance of implantation regarding the establishment of pregnancy becomes evident by the illustration of the "pregnancy loss iceberg"[12,13] (Figure 1.3) which visualizes that 55% of spontaneous and 65% of IVF pregnancies are lost pre-clinically (i.e. prior to the time of missed menstruation and therewith normally unperceived) due to implantation failure.

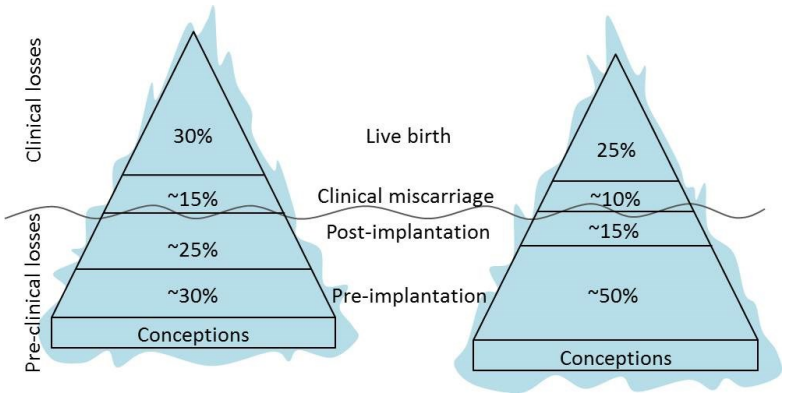

Fig. 1.3: Pregnancy loss iceberg. Percental distribution of pregnancy outcomes after spontaneous conception (left) and IVF-treatment (right). The water surface discriminates "invisible" pre-clinical pregnancy losses from clinical evident pregnancy losses.

If fertilization of the oocyte occurs in the fallopian tube, the conceptus migrates to the uterus within 4 days and passes through different stages of development until it arrives at the uterine wall as a blastocyst. The blastocyst consists of an inner cell mass, the embryoblast and the cell-free blastocoel, which is encircled by a layer of cells, the designated trophoblast, and a protective glycoprotein membrane, the *zona pellucida* (Figure 1.4).

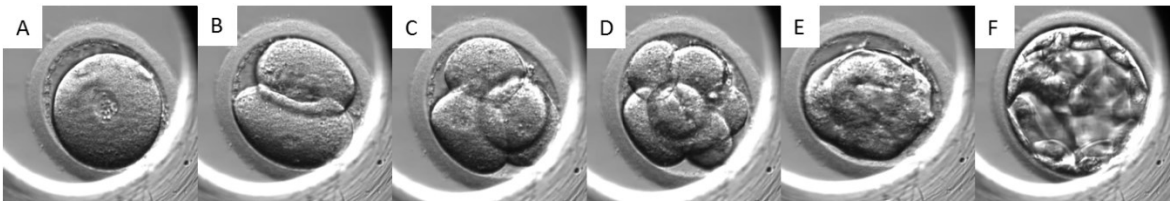

Fig. 1.4: Human embryonic development from (A) pronuclear stage, (B) 2-cell-stage, (C) 4-cell-stage, (D) 8-cell-stage and (E) compacted morula to (F) blastocyst.

After contact with the receptive endometrium, the blastocyst leaves its *zona pellucida* ("hatching") and a loose contact between blastocyst and endometrium is generated in the first stage the "apposition", of implantation. Thereafter, the outer trophoblastic cells facilitate the interaction with the EECs in the second stage referred to as adhesion. The final stage of implantation is the invasion of the in embryo into the maternal endometrial tissue, which is accompanied by the differentiation of the trophoblast into two cell populations: the

inner cytotrophoblast built of mononuclear cells surrounding the inner cell mass, representing the place of intense cell division, and the peripheral syncytiotrophoblast, a multinucleated layer of merged cells[14,15]. The invasive syncytiotrophoblast disrupts the endometrial epithelium and invades into the stroma as far as the proximal third of the myometrium. A process, which seems to be rather distinctly regulated in space and time[16,17]. The whole process of implantation is completed after 14 days. After this time the entire embryo is surrounded by maternal tissue. The syncytiotrophoblast meanwhile facilitates the affiliation to the maternal vascular system by rupturing and lining the maternal uterine capillaries[7,18].

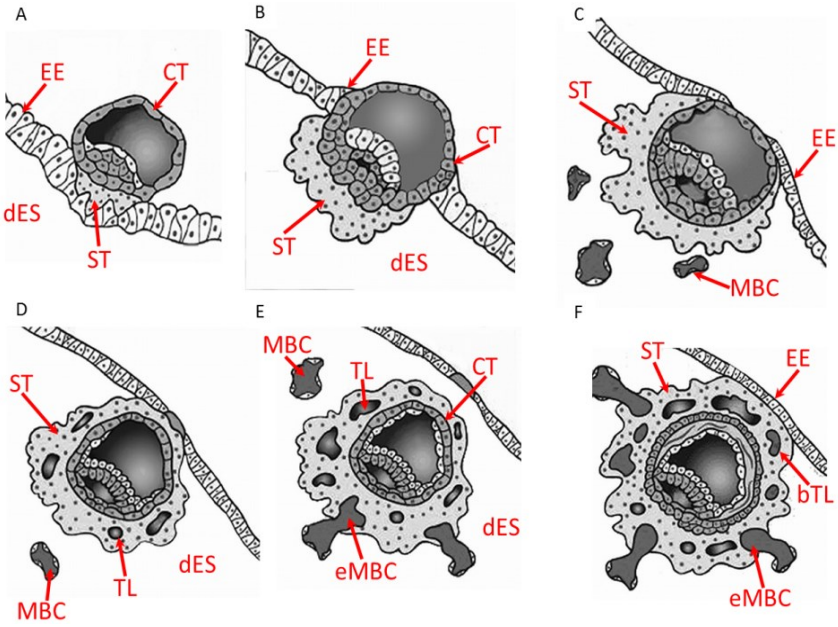

Fig. 1.5: Stages of the embryo implantation: after apposition of the cytotrophoblast [CT] to the endometrial epithelium [EE], the syncytiotrophoblast [ST] invades the decidualized endometrial stroma [dES] which is rich in maternal blood capillaries [MBC]. With proceeding embryonic invasion extracellular caves, named trophoblastic lacunae [TL] appear in the ST which are filled with blood [bTL] after the MBCs are eroded [eMBC]. Modified after[143]

Taken together the embryo implantation (Figure 1.5) as a crucial step for subsequent successful pregnancy requires a receptive endometrium with secretory glands and dESCs[8], a good quality embryo at the blastocysts stage, and a synchronized embryo-maternal dialog[19], conducted by secreted cytokines, chemokines and growth factors as well as expression of corresponding receptors and co-receptors[20,21].

Cytokines are a broad class of small proteins, which are secreted by a vast amount of different cells, in contrast to hormones which are only produced by a few specific cells. Cytokines affect cells and their function by binding to specific receptors which thereby influence intracellular signaling pathways. Cytokines, which particularly mediate a directed movement of cells (chemotaxis) are referred to as chemokines. Growth factors are also secreted proteins, which act as signaling molecules by binding to receptors. Sometimes the terms cytokines and growth factors are used synonymously. It should be noted that most

growth factors are cytokines but on the contrary not all cytokines are growth factors due to the fact that growth factors mediate cell growth and proliferation in accordance to their name, while cytokines may also inhibit proliferation or even induce apoptosis[22–24].

Cytokines secreted by endometrial and trophoblast cells have already been described to decisively regulate every step of implantation[25]. More detailed, high levels of interleukin (IL)-1β are released by human embryos *in vitro*[26,27]. Interferon (IFN)-γ is expressed by the early trophoblast and endometrial, immune cells whose number rapidly expand in early pregnancy[28]. Tumor necrosis factor (TNF)-α and the corresponding receptors are found in endometrium, placenta and the fetus during pregnancy[29]. Furthermore, transforming growth factor (TGF)-β1 is expressed in endometrial stromal and epithelial cells [30] and in the syncytiotrophoblastic layer[31]. Early studies from our laboratory revealed that decidualized stromal cells highly upregulate chemokines such as CXCL1, IL-8 and CXCR4 after treatment with embryonic secretion products[20]. Taken together, secretion of diverse cytokines and expression of the corresponding receptors provide the basis for a dynamic and well synchronized embryo-maternal dialog during implantation.

1.3 Pregnancy disorders

Dysfunction of the embryo invasion as a tight regulated procedure can cause insufficient implantation, which in turn enables the occurrence of several known diseases possibly leading to developmental disorders or even threaten maternal and/or embryonic life.

Examples of pregnancy disorders are preterm delivery (birth of the baby before 37 weeks of gestational age), intrauterine growth restriction (IUGR; poor growth of the fetus during pregnancy) and equally important preeclampsia, which is characterized by maternal high blood pressure and protein occurrence in the urine and which can lead to life-threatening eclampsia[32]. Furthermore as the HELLP syndrome, a life threatening disorder which is characterized by hemolysis (H), elevated liver enzymes (EL) and low platelet count (LP)[33] may be induced. These disorders have in common that they are mostly allocated to shallow implantation[34,35].

In this context it should be discussed whether techniques of ART correlate with the risk of a deficient implantation and therefore are associated with subsequent pregnancy disorders. To my knowledge, no study exists in the current literature, which connects the coherence of an IVF treatment and the risk of implantation failure directly. Nevertheless,

several studies describe an increased occurrence of adverse pregnancy outcomes in those women who conceived after IVF treatment, including preterm delivery, low birth weight (which might be connected to IUGR), preeclampsia, and *placenta previa*, an atypical/cervical localization of the placenta[36–39]. In contrast, authors of another study criticized that disruptive factors, including especially age, were not incorporated in these statistical evaluations. Therefore, this group performed a special analysis to minimize confunders, such as age, re-examined the risk for preeclampsia in pregnancies after IVF treatment and failed to find any correlation. In summary, they concluded that the association between IVF therapies and preeclampsia shown by other studies is swayed by many factors and therefore should not be connected[40]. Herein, it should be considered that more women of advanced age, which was defined being >35 years old at the time of delivery[41], undergo IVF treatments compared to younger women[42]. A higher maternal age was already correlated with an increased risk of pregnancy disorders, enclosed preeclampsia, *placenta previa,* low birth weight, and miscarriages[43–46]. This is important since the average age of primipara gradually increased over the last 20 years in Germany[47]. Therefore, investigations are urgently needed to first achieve a comprehensive understanding of the process of implantation and secondly to improve techniques of ART to support a proper implantation. This would help to prevent obstetrical problems and particularly long-term side effects for children achieved by ART methods.

1.4 Apoptosis – the programmed cell death

The term “apoptosis” describes a process of eliminating single cells without an immune response and damage of the surrounding tissue. It was first introduced in 1972 when the distinctive morphological changes (fragmentation and enfolding of cell compartments into membrane-covered apoptotic bodies that were removed via phagocytosis) were documented by electron microscopy[48]. More precisely, apoptotic cells reveal an initial shrinkage followed by detachment from the surrounding tissue. Thereafter, nuclear chromatin condensates and the DNA is degraded into internucleosomal fragments by endonucleases[49]. The plasma membrane bulges outwards and loses its asymmetry as the cytosolic membrane component phosphatidylserine translocates to the outer surface of the cell[48,50]. Membrane blebs separate from the cell and form membrane covered particles (apoptotic bodies), which contain cellular organelles and fragments of the nucleus. These are

phagocytosed by neighboring cells, at which phosphatidylserine serves as an “eat me”-signal at the surface[51].

Apoptosis critically governs several different physiological processes such as separation of fingers and toes during the embryo development[52], elimination of self-reactive lymphocytes for achieving self-tolerance and immune homeostasis[53] and maintaining the homeostatic cellular balance as an antagonist to mitotic cell proliferation[54].

Correspondingly, dysregulated apoptosis can provoke miscellaneous indispositions: on the one hand insufficient apoptosis can cause autoimmune diseases, cancer and virus infections, on the other hand exaggerated apoptosis can influence the pathology of AIDS, apoplectic stroke and neurodegenerative diseases such as Parkinson and Alzheimer’s[55–58].

Apoptotic cell death has a momentous influence on the organism and is therefore tightly and multilayered regulated by pivotal mediators, cysteine-dependent aspartate-specific proteases, called caspases. By means of their cellular position and role in apoptosis, caspases can be classified into two groups: initiator caspases (Caspase-8 and -9) which are activated in multicomponent protein-complexes and further activate downstream effector caspases (Caspase-3) which irreversibly triggers the cell death with its aforementioned morphological changes[59]. The death signal attains the cell either via external ligands or internal stressors. The extrinsic pathway is initiated by death receptors (R), i.e. FasR or TNF-related apoptosis-inducing ligand receptor (TRAIL R), leading to conformational changes of the receptor and recruitment of adaptor proteins in a multicomponent complex, in which Caspase-8 is activated[60]. The intrinsic pathway is initiated via various stimuli e.g. oxidative stress, DNA damage or growth factor deprivation, which leads to the permeabilization of mitochondria and a release of Cytochrome C, which aggregates with proteins in a complex and afterwards activates Caspase-9[61]. Subsequently, both pathways lead to the activation of Caspase-3 and are regulated by several pro- and anti-apoptotic factors. The Inhibitor of Apoptosis (IAP) family includes different members (XIAP, cIAP-1, -2, Survivin and Livin), which directly inactivate caspases[62]. Furthermore, cIAP-1 and -2 and XIAP serve as suppressors of Fas-mediated apoptosis through Caspase-3 inhibition and facilitate the transcription of pro-survival genes via activating nuclear factor (NF)κB[63]. On the contrary, pro-apoptotic molecules for instance the Second mitochondria-derived activator of caspases (SMAC) and the High temperature requirement protein A2 (HtrA2), both released from the mitochondria bind IAPs and either reduce or even prevent their inhibitory effects on apoptosis[64]. Members

of the B-cell lymphoma 2 (Bcl-2) family govern different actions regarding apoptosis, they either facilitate (Bad, Bax) or prevent (Bcl-2, Bcl-xL) the permeabilization of mitochondria and therewith function in a pro- or anti-apoptotic manner.

The complex network of interacting pro- and anti-apoptotic proteins is well-adjusted in a viable cell, but a death signal leads to a shift to pro-apoptotic proteins, whose impact is no longer buffered by the corresponding pro-survival proteins and consequently leads to apoptosis.

Cell death as an irreversible and far-reaching incident is mediated via signaling pathways including mitogen-activated protein kinases (MAPKs) and the proteinkinase Akt. Pro-apoptotic MAPKs, such as JNK, exist in an inactive form within the cell. They need a multi-step pathway of diverse phosphorylation events in response to different cell stressors including cytokines, to become activated. Activated MAPK can directly interact with other apoptosis-related proteins, inhibit pro-survival properties and facilitate the transcription of pro-apoptotic genes[65,66]. Correspondingly JNK directly inhibits anti-apoptotic proteins e.g. Bcl-2 and Bcl-xL, promotes pro-apoptotic Bad and Bax and lead to the expression of pro-apoptotic genes implying Bak and Fas-L indirectly by activating the transcription factor c-Jun[67]. Akt is a popular key regulator of the phosphatidylinositol-3-kinase (PI3K)-signaling pathway and influences multiple cellular processes. Several steps are required to activate Akt. Ligands, such as growth factors and other cytokines, stimulate specific transmembrane receptors, which activate PI3K. Further, Akt is recruited to the membrane and interacts with a kinase named PIP_3, which leads to an alteration of the Akt conformation, a crucial requirement for phosphorylation and activation of Akt[68]. The protein Akt possesses several phosphorylation sites whose functions are mostly not elucidated until today. Nevertheless, it is known that Akt needs to be phosphorylated at two sides to achieve its complete kinetic activity[69] and respectively dephosphorylation at both sides leads to an inactivation of Akt. Up to date approx. 100 target proteins of Akt are identified, which mediate cell proliferation, -cycle, -growth and -survival. The latter is promoted via direct inhibition of pro-apoptotic proteins including Bad and indirectly via blocking the transcription of forkhead box (FOX)O-mediated pro-apoptotic target genes and the pro-apoptotic signaling pathways enclosing JNK[70]. Furthermore, Akt activates anti-apoptotic XIAP, Survivin and pro-survival transcription factors like NFκB, which subsequently induces the expression of IAPs[71,72].

1.4.1 Apoptosis in the human reproductive tract

An important role of apoptosis was already described for several physiologic processes in the human female reproductive tract. Inter alia, the distinct reduction in number of ovarian follicles present at birth to adolescence and later the huge amount of follicles needed monthly to mature one follicle for ovulation, was associated with apoptotic follicle death in all different developmental stages[73].

In connection with the uterine menstruation cycle, in particular with the menstrual breakdown of the endometrium, the involvement of apoptosis was intensely investigated and described in the present literature. The different stages (as described in chapter 1.1) are characterized by a varying occurrence of apoptosis in connection with a specific expression of apoptosis related proteins. As one might imagine, there is nearly no apoptosis present in the endometrium in the proliferative phase. But multiple studies pointed out that at the beginning of the secretory phase apoptotic endometrial glands and stromal cells appear and the number rises throughout the receptive and following late secretory phase until almost all components of the functional layer are involved during menstruation[74–76]. Because of these observations the role of apoptosis as a mediator for endometrial breakdown during the menstrual shedding is conceivable and generally accepted. Nonetheless, other antithetic studies exist, in which on the one hand the presence of only a small number of apoptotic stromal cells throughout the cycle[76,77] is described or on the other hand an almost equal quantity of apoptotic stromal cells at every phase of the cycle[78].

Nevertheless, the expression of apoptosis-related factors in the endometrium in conjunction with the phase of menstrual cycle was also highly examined. The surface molecules FasR and TNF R seem to be expressed constantly[79], but FasR was observed to be localized in the Golgi apparatus during the proliferative phase of the cycle translocating to the cell membrane during the secretory phase[80]. Furthermore, the corresponding ligands FasL and TNF are expressed as a function of cyclic-/hormone-dependent changes in the endometrial stroma and epithelium, which is mirrored by an increase of the ligands during the secretory phase[79,81]. Accordingly, a strong expression of Bcl-2 was observed during the proliferative but not late secretory phase, indicating a role of this anti-apoptotic protein in protecting endometrial cells from apoptosis during the proliferative phase[82]. Further references, indicating a role for the intrinsic and extrinsic apoptotic pathway in regulation of the menstrual tissue breakdown state an upregulation of active Caspase-3, -8 and -9 in

endometrial cells during the late secretory phase[83] accompanied by an increase of pro-apoptotic Bax[84].

In early pregnancy the blastocyst expresses different factors including cytokines, growth factors, FasL and TRAIL, which are supposed to be responsible for the induction of apoptosis of activated decidual immune cells to maintain immune privilege protecting the fetal allograft from the maternal immune system[85].

1.4.2 Implantation associated apoptosis in human and animals

The exact cellular mechanisms mediating a proper implantation in human are still not fully understood, but in recent years, the interest of endometrial apoptosis as a fundamental prerequisite for successful pregnancy gained more and more interest. Up to date the literature regarding the role of endometrial apoptosis during embryo implantation is discrepant: on one hand the EEC apoptosis after blastocysts adhesion was intensely explored, whereas on the other hand studies about human ESC apoptosis are rare and most information derives of animal studies. Furthermore, even antithetic literature exists describing ESC apoptosis on the one hand and a resistance of ESCs towards apoptosis on the other showing the uncertainty of the current knowledge and the need of elucidating investigations.

Trophoblast penetration of the endometrial epithelial layer as the earliest event of human embryo implantation was already investigated before. It was revealed that the embryo bears FasL and endometrial cells the corresponding receptor FasR, in connection to that EEC disruption was accompanied by Fas-mediated apoptosis as a consequence to trophoblast-endometrial contact during the adhesion phase of the implantation process[86–88]. Subsequently, *in vitro* co-culture experiments were performed with human trophoblast spheroids (globular cell conglomerates) attached to an EEC monolayer. Spreading of the spheroid in accordance to apoptosis at the spheroid-EEC interface was observed[89]. Treatment with an anti-Fas antibody (ab), which activated the FasR, led to an increased spheroid expansion and enhanced EEC apoptosis, whereas a FasR neutralizing ab prevented spheroid outgrowth[88].

Although results of animal studies are not 100 percent transferable to the human system, it needs to be discussed when it comes to ESC apoptosis since little data about human are currently available. Correspondingly, animal studies revealed an important role

of EEC and ESC apoptosis in the establishment of pregnancy in mice and rats[90–93] as well as in rhesus monkeys[94] and dogs[95]. A rather detailed study analyzed the regulation of apoptosis in early stages of murine pregnancy in ESCs compared to EECs and the authors concluded that two different signaling pathways are involved. The rapid and complete disruption of the epithelial layer is mediated via the extrinsic apoptotic pathway. In contrast dESC apoptosis is regulated in a more moderate spatial and temporal fashion conciliated by the intrinsic pathway[96] and this occurrence probably controls the aforementioned precise extent of invasion as a crucial factor for a successful pregnancy.

In vivo studies about human ESC apoptosis during implantation are rare. *In vitro* studies described an inveterate resistance of ESCs toward apoptosis independent of the cells being non-differentiated or decidualized[97–99]. Nevertheless, ESC resistance toward Fas-mediated apoptosis was overcome by pretreatment of the cells with interferon (IFN)-γ and tumor necrosis factor (TNF)-α[97]. Furthermore, it was shown before that transforming growth factor (TGF)-β1 induces apoptosis in primary cultured ESCs[100]. Other studies described the role of heparin-binding (HB)-EGF, which is expressed by dESCs and trophoblast cells[101], mediating the survival[102,103] and *in vitro* motility of dESCs[104], suggesting an rather active supportive role of dESCs during the implantation instead of a passive induction of apoptosis.

Several clinical studies revealed that multiple pregnancy complications and disorders are associated with altered placental development due to apoptosis compared to normal developed placentas. An increased number of apoptotic endometrial and placental cells was shown in cases of miscarriages[74] and unexplained recurrent spontaneous abortion (both terms describe the death of the embryo or fetus in the uterus)[105], preeclampsia[106–108], HELLP-Syndrome[106,107,109], IUGR[106,110–112] and in *endometrium out of receptive alignment* (a syndrome which is related to repeated implantation failure and consecutive infertility or early pregnancy loss)[113], whereas a diminished number of apoptotic cells was observed as a consequence of ectopic pregnancy outside the uterus[114].

Taken together current literature regarding endometrial apoptosis as a crucial step of embryo implantation turns out to be very divergent: on one hand EEC apoptosis induced by the embryo contact was keenly researched, whereas on the other hand studies about human ESC apoptosis are rare and rather conflicting describing either the existence or resistance of apoptosis within these cells.

Another very important aspect during implantation is the remodeling of maternal spiral arteries, which ensure the nutrition of the embryo at this early phase of pregnancy. As already mentioned in chapter 1.1 the spiral arteries develop during the secretory phase within the endometrium. During implantation these vessels are strongly dilated and their contact with the invasive syncytiotrophoblast leads to the loss of vascular smooth muscle cells and endothelial cells and correspondingly causing a blood flow out of the eroded vessel into lacunae inside the syncytiotrophoblast (see also Figure 5). Different studies correlate the vascular cell destruction with the apoptotic cell death facilitating spiral artery remodeling. For example, it was shown that trophoblast-derived secretion products activate the FasR and TRAIL R system in endothelial and vascular smooth muscle cells *in vitro* and further induce apoptosis[115,116]. *In situ* studies of first trimester placental bed samples exhibit divergent results, namely the presence [117] and absence [18] of apoptotic vascular smooth muscle cells as a consequence to spiral artery remodeling. The nonexistence of apoptosis in the latter study was correlated with an active vascular cell migration away from the artery. But other studies point out that the rapid removal of apoptotic cells via a large number of phagocytes within the decidua aggravates the possibility to detect apoptosis[7,117].

1.5 Syndecan-1

Syndecan-1 (Sdc-1) as schematically displayed in Figure 1.6 is a transmembrane proteoglycan, i.e. a protein, which is present on the cell surface, consisting of a cytoplasmatic, a transmembrane and an extracellular core domain, which typically carries chains of mainly heparan sulfate (HS) but also chondroitin sulfate (CS)[118,119]. Sdc-1 acts as a co-receptor and storage molecule for a wide range of ligands including cytokines and growth factors. Its HS chains affect the binding, stability and conformation of ligands and their corresponding receptors or increase the ligand concentration on the cell surface. Additionally, different studies proposed a role of the highly conserved cytoplasmatic domain, regarding the interaction with cytoskeletal proteins and downstream signal transducers and therefore the responsibility for mediating a non-catalytic/mechanical transduction, although the molecular mechanisms are not known[120,121]. In this context it was further described, that alterations of the Sdc-1 expression lead to changes regarding cell morphology, growth and migration, which are all processes connected to cytoskeletal organization[121].

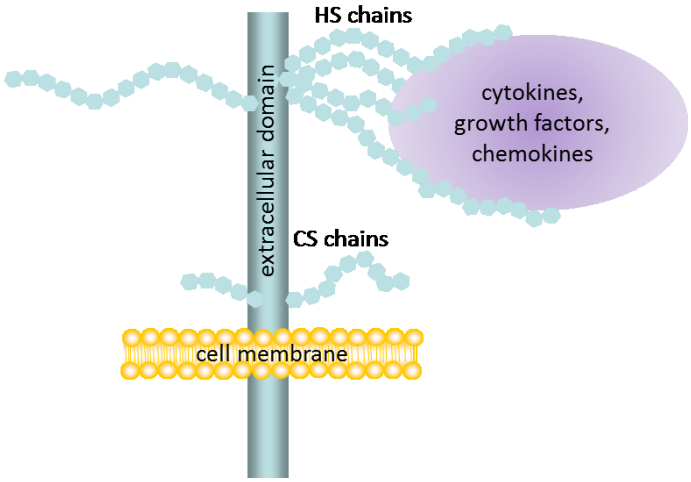

Fig. 1.6: Schematic depiction of Syndecan-1, a cell surface proteoglycan, containing chains of heparan sulfate (HS) and chondroitin sulfate (CS) and associated binding ligands such as cytokines and growth factors.

Besides, cytoplasmatic Sdc-1 was found to be a microtubule-associated protein in the mitotic spindle of dividing cells, indicating a role of Sdc-1 in mediating cell proliferation[122]. The ability of Sdc-1 to interact with tubulin is also linked to the findings that Sdc-1 translocates to the nucleus and is assumed to modulate the function of transcription factors therein[122,123].

Moreover, proteolytical cleavage of intact Sdc-1 ectodomains from the cell surface, a process recognized as *shedding,* via heparanase or matrix metalloproteinases (MMP), leads to soluble effectors in the extracellular milieu and body fluids[124]. In this context a study with MMP and Sdc-1 knock out (ko) mice revealed that chemokines bound to Sdc-1 are important chemoattractants for immune cells, e.g. neutrophils. Shedding of these chemokine-Sdc-1 complexes influences whether the recruitment of immune cells occurs on the cell surface or in the extracellular milieu[125]. Furthermore, a study with primary human umbilical vein endothelial cells (HUVEC), revealed that membrane bound chemokine-Sdc-1 complexes facilitate the transendothelial migration of neutrophils, whereas shedding of these complexes destroys the chemotactic gradient resulting in a decreased immune cell transmigration[126]. These results indicate an important role of Sdc-1 and particularly Sdc-1 shedding in implantation-related processes, including inflammation and angiogenesis.

Taken together, the supposed biological functions of Sdc-1 are rather multiple and complex and comprise regulation of cell-cell-interaction, cell proliferation and –differentiation, embryonic development, angiogenesis, and tumor development.

1.5.1 Syndecan-1 in the reproductive biology

Sdc-1 attracted interest in the field of obstetrics and gynecology as well as reproductive medicine in the recent years. It was identified in the female reproductive tract (vaginal epithelium, cervix, and endometrium) of normal cycling healthy women. The expression pattern varies throughout the menstrual cycle, implicating an influence of sex steroids on the regulation of Sdc-1, although the corresponding literature demonstrates an inconsistency.

One study describes Sdc-1 in the whole endometrium (luminal and glandular epithelium, as well as stroma) with diverse and partly opposed expression patterns. More specifically, in the luminal epithelium Sdc-1 was significantly decreased during the proliferative phase, whereas no changes were observed for the glandular epithelium throughout the cycle. Opposite to the decreasing luminal expression in the proliferative phase the stromal expression of Sdc-1 increased in the proliferative phase[127]. However, another group found an increase of Sdc-1 mRNA expression during the secretory phase of the cycle in the entire endometrial tissue and immunohistochemistry analysis revealed that Sdc-1 was mainly localized at the apical surface of the glandular epithelium during the phase of endometrial receptivity, the so-called window of implantation[128]. Accordingly, another group also described a rise of endometrial epithelial Sdc-1 in the secretory phase[129]. In summary, regulation of Sdc-1 via sex steroids with an increase in the secretory/receptive phase epithelium is conceivable, whereas the current literature propagates the contrary effect in the stroma with a Sdc-1 decrease in the secretory phase of the cycle. Interestingly, one study regarding Sdc-1 expression in the placenta and decidua at early pregnancy demonstrated an intense expression of Sdc-1 in the syncytiotrophoblast and placenta, but failed to detect Sdc-1 in the decidualized stroma and glands[130]. Studies in mice revealed a Sdc-1 expression already in four-cell stage embryos, which persisted until the late blastocyst stage[131]. Correspondingly, human syncytiotrophoblasts exhibited a strong Sdc-1 expression at the apical surface[130] indicating a potential role of Sdc-1 in the embryo-maternal dialog, a prerequisite for a proper implantation, as mentioned, even though the exact functions of Sdc-1 in the human placenta are still not fully understood.

Clinical studies were performed to clarify the involvement of Sdc-1 in the development of pregnancy disorders, which result from inadequate implantation as described in chapter 1.3. In all outlined complications an altered placental Sdc-1 expression compared to healthy

controls was found. But regarding the exact correlation of Sdc-1 and disease occurrence Sdc-1 revealed a Janus-faced attitude and therefore the findings were shown to be extremely divergent. On the one hand, reduced Sdc-1 expression was correlated with intrauterine growth restriction[132], preeclampsia[133] and HELLP syndrome[134] and accordingly elevated Sdc-1 was connected with a reduced risk for preterm labor[135]. But on the other hand increased syncytiotrophoblastic Sdc-1 expression was also associated with preeclampsia and HELLP syndrome[136]. The limitation of these studies and studies of the human reproductive biology in general is the restricted disposability of samples from patients with pregnancy diseases as well as healthy controls in early pregnancy stages. Therefore, the current studies in the available literature are not able to comprehensively depict Sdc-1's role in the establishment of the aforementioned pregnancy diseases and detailed investigations are urgently needed.

1.5.2 Syndecan-1 and apoptosis

Studies describing an influence of Sdc-1 regarding the regulation of apoptosis in cancer cells are abundant. In this context a Janus-faced attitude of Sdc-1 was also observed. However, these findings are oppositional and are implying that Sdc-1 has a varying impact on the development and malignancy of cancer. In terms of tumor cells of the human female reproductive tract, it was described that Sdc-1 expression was decreased in invasive cervical carcinoma cells compared to healthy cervical cells and the inverse correlation between Sdc-1 expression and development of metastasis was shown[137]. In contrast, an increased Sdc-1 expression was correlated with high proliferation, invasion, aggressive phenotype and poor clinical prognosis for breast[138] and ovarian[139] cancer. In endometrial cancer an elevated Sdc-1 expression was observed compared to healthy endometrium. Furthermore, overexpression of Sdc-1 in endometrial cancer cells via *in vitro* transfection promoted cell proliferation[140], whereas silencing of Sdc-1 induced apoptosis[141].

In spite of the contrary literature regarding Sdc-1's role in cancer and pregnancy diseases, an influence of Sdc-1 on endometrial cell apoptosis during the physiological process of implantation is conceivable, although the knowledge about the exact mechanism is very limited so far.

1.6 References

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2. Aim of the study

The purpose of this study was to clarify the role of the female endometrium in facilitating the establishment of a successful pregnancy, especially the physiological role of endometrial cell apoptosis during the process of embryo implantation. In particular the apoptotic inducibility of ESCs was supposed to be further elucidated, because it has not been sufficiently studied before and a lot of questions remained unclear which are important for the field of reproductive medicine. Although implantation-associated EEC apoptosis attracted more attention in the past, these cells were also studied herein to clarify the conflicting literature and to achieve comparability between the epithelium and the stroma composing an interconnected organ *in vivo*.

Furthermore, a possible role of Sdc-1 facilitating a proper implantation via mediating the apoptotic signal in EECs and ECSs should be resolved using a stable Sdc-1 knock down (kd), which was generated in ESCs before in our laboratory and should be reproduced in EECs within the scope of this study. To gain insight into a possible role of implantation-related endometrial apoptosis a suitable model of embryo contact during implantation was supposed to be established. For this purpose embryo secretion products and surface molecules as an *in vitro* treatment of the cells before investigating the induction of apoptosis and corresponding signaling pathways were applied.

Taken together the results of this study should provide further insight into the complex and momentous process of implantation deciphering an important role for maternal endometrial cell apoptosis to successfully establish a pregnancy. The impact of Sdc-1 in regulating endometrial apoptosis is particularly interesting in the light of an already described influence on pre-clinical pregnancy losses, pregnancy complications and disorders, and therefore might provide a useful clinical tool in the future to prevent infertility or pregnancy complications caused by insufficient implantation.

3. Articles

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

Original Research Article

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molecular human reproduction

ORIGINAL RESEARCH

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.

Key words: 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 (Schlafke *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 tight 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 in the extracellular milieu (Götte and Echtermeyer, 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 phase (Germeyer *et al.*, 2007). Furthermore, an influence of Sdc-1 knockdown (kd) in protein expression pattern of cytokines and angiogenic factors was published before (Baston-Büst *et al.*, 2010).

Recent publications about the influence of Sdc-1 on apoptosis revealed a Janus-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; Numa *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% CO_2 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, Steelze, Germany).

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®, 5×10^5 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, 4×10^5 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 Blasticidin 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′-GCT TTG CTC GAC GCC TTTAG-3′ and rev 5′-GGT GTT TCC CTT TCT TCT TTA 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 Invitrogens' RNAi designer, following shRNA oligonucleotides for the Sdc-1 kd were designed: top strand 5′-CAC C**GC AGG TGC TTT GCA AGA TAT C***CG AA*G ATA TCT TGC AAA GCA CCT GC-3′, bottom strand 5′-AAA A**GC AGG TGC TTT GCA AGA TAT C***TT CG*G ATA TCT TGC AAA GCA CCT GC-3′; Sdc-1 database sequence AJ551176.1, Location: 393 **GCA GGT GCT TTG CAA GAT ATC** 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 (Duesseldorf, 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 Invivogen). Successfully double-transfected cells contain the TetR protein encoding vector pcDNA™/TR and the vector pENTR™/H1/TO encoding the Sdc-1 shRNA downstream of the H1/TO pol III promoter which contains Tet-Operator 2 ($TetO_2$) binding sites for Tet-regulated expression. Without Tet the TetR binds the $TetO_2$ sites and blocks the shRNA transcription. After addition, Tet binds the TetR leading to conformation changes and subsequent to release from the $TetO_2$ sites which induces the transcription of 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 $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers for Sdc-1 were for 5′-GGAGAATACGGCTG TAGTGG-3′ and rev 5′-AGCTGCCTTCGTCCTTCGTTC-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 buffer soaked Whatman paper with a methanol pre-treated PVDF membrane (Merck Millipore, Billerica, MA, USA) on top was layered. Thirty-microgram protein of Tet-induced and not induced transfectants was applied to the membrane. After drying, the membrane was blocked with 5% non-fat milk powder (Sigma-Aldrich) for 1 h and incubated with 0.5 μg/ml rabbit anti-human Sdc-1 ab (ab60199, Abcam, Cambridge, UK) for 2 h followed by goat anti-rabbit HRP ab (R&D Systems, Minneapolis, MN, USA) for 1 h. Signals were detected using RapidStep™ ECL Reagent (Calbiochem, Darmstadt, Germany) and analyzed with an Alpha Imager camera (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). The transfectant with the lowest signal for Sdc-1 after induction with Tet was chosen for further experiments and named RLSdc1kd.

Active Caspase-3 measurement

Expression of active Caspase-3 was analyzed applying the Quantikine® ELISA (R&D Systems). RL95-2 and induced RLSdc1kd cells grown in 24-well plates were treated with 5 μg/ml anti-human CD95 (Fas) ab clone EOS9.1 (Biolegend, San Diego, CA, 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 (all Biolegend) for 24 h alone or additionally with 5 μg/ml anti-human CD95 (Fas) ab for another 7 h. Cell extract preparation and measurement of active Caspase-3 was performed according to the manufacturer's protocol and given as relative amount of active Caspase-3 in ng/ml.

Active Caspase-8 and -9 measurements

Enzymatic activity of the Caspases-8 and -9 after activation was determined via colorimetric assays (R&D Systems) according to the manufacturer's manual. RL95-2 and 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 and compared with non-treated controls. The enzymatic activity was calculated as fold induction compared with control.

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 by 12% SDS–PAGE and transferred to 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).

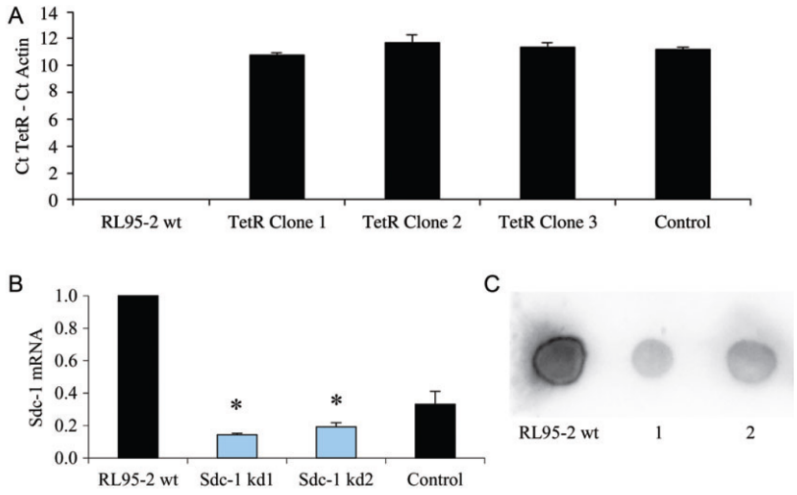

Figure 1 Syndecan-1 (Sdc-1) knockdown (kd) in human endometrial epithelial carcinoma cell line RL95-2. (**A**) Bacterial TetR mRNA expression of transfected RL95-2 clones. Shown are the ΔCt of TetR template less the housekeeping gene β-Actin. For untransfected RL95-2 the Ct of TetR was given as >40 cycles. Successfully transfected and published stroma cells served as control (Baston-Büst *et al.*, 2010). Data represents means ± SEM of three independent experiments. (**B**) Sdc-1 mRNA expression after Tet-induction. Double-transfected RL95-2 clones were induced with 1 μg/ml Tet for 24 h. Fold induction of Sdc-1 mRNA in two clones after induction (light blue) versus not induced cells with wildtype Sdc-1 (RL95-2wt; black). Successfully double-transfected and published stroma cells served as control (Baston-Büst *et al.*, 2010). Data represents means ± SEM of five independent experiments, $^{*}P < 0.05$ wt cells versus Sdc-1 kd clones. (**C**) Protein dot immunoblot with 30 μg membrane protein fraction of two clones (1, 2) after Tet-induction versus not induced cells with wt Sdc-1.

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).

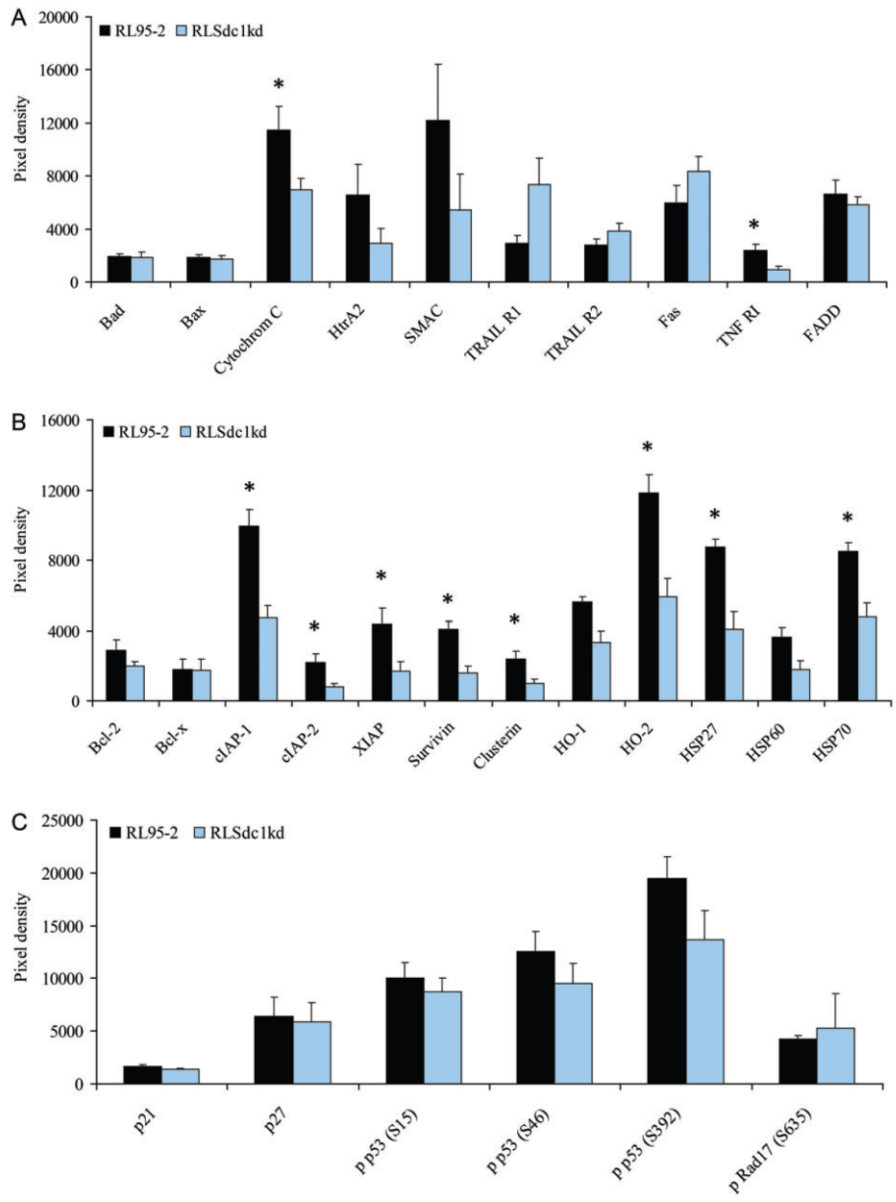

Figure 2 Apoptosis Ab Array of untreated control RL95-2 versus RLSdc1kd. Shown are the pixel densities of different apoptosis-related proteins divided into groups according to their cellular function. (**A**) Pro-apoptotic intrinsic proteins, extrinsic death receptors and adapter molecules. (**B**) Proteins 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.

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

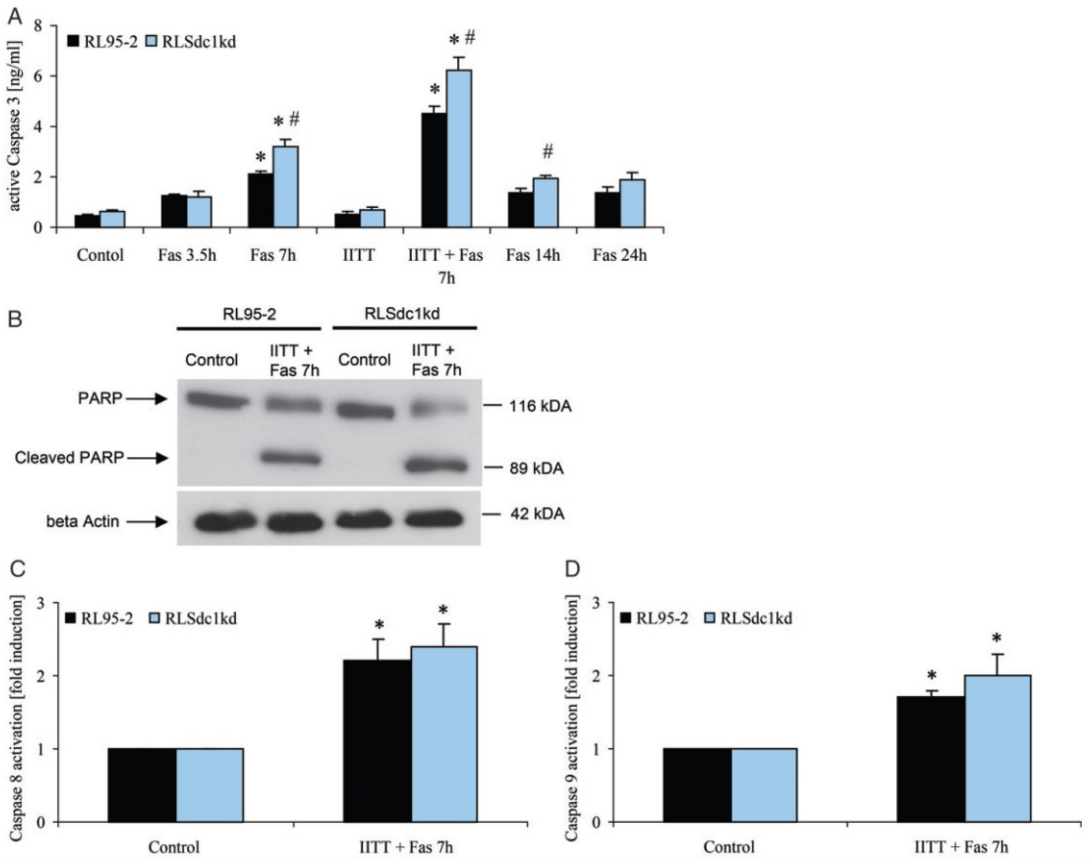

Figure 3 Analysis of apoptosis in EECs. (**A**) Measurement of active Caspase-3 (ng/ml) in RL95-2 (black) and Tet-induced RLSdc1kd (light blue). Cells were treated with 5 μg/ml anti-human Fas ab for 3.5, 7 and 14 h or with 1 μg/ml for 24 h. Furthermore, with a combination of 10 ng/ml IL-1β, 10 ng/ml IFN-γ, 5 ng/ml TNF-α, 0.5 ng/ml TGF-β1 (IITT) for 24 h alone and additionally with 5 μg/ml anti-Fas ab for another 7 h, untreated cells served as control. Data represent means ± SEM of 4–5 independent experiments. *$P < 0.05$ treated cells versus untreated control, #$P < 0.05$ RL95-2 versus RLSdc1kd. (**B**) Western blot analysis of PARP and cleaved PARP after treatment with a combination of IITT (24 h) and anti-Fas ab (7 h) compared with untreated control. β-Actin served as loading control. (**C** and **D**) Induction of active Caspase-8 and -9 after treatment with combination of IITT (24 h) and anti-Fas Ab (7 h). Data represent means ± SEM of four independent experiments. *$P < 0.05$ treated cells versus untreated control.

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 1 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

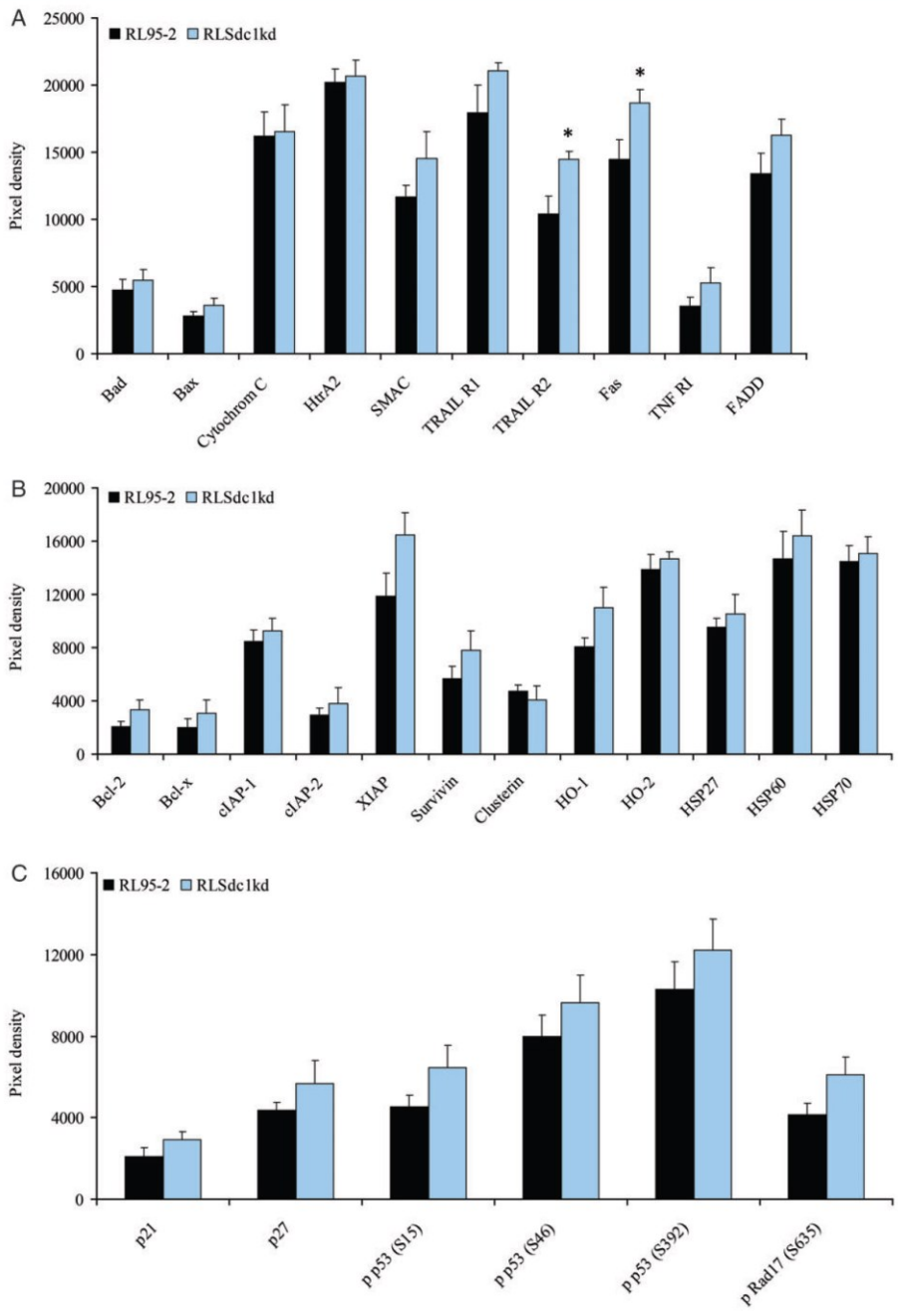

Figure 4 Apoptosis Ab Array of RL95-2 and RLSdc1kd after treatment with a combination of 10 ng/ml IL-1β, 10 ng/ml IFN-γ, 5 ng/ml TNF-α, 0.5 ng/ml TGF-β1 (IITT) for 24 h and additionally with 5 μg/ml anti-Fas ab for another 7 h. Shown are pixel densities of different apoptosis-related proteins divided 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.

Table I 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).

	Control versus treated	
	RL95-2	RLSdc1kd
Bad	*	*
Bax		*
Cytochrom C		*
HtrA2	*	*
SMAC		
TRAIL R1	*	*
TRAIL R2	*	*
Fas	*	*
TNF R1		*
FADD	*	*
Bcl-2		
Bcl-x		
cIAP-1		*
cIAP-2		*
XIAP	*	*
Survivin		*
Clusterin	*	*
HO-1	*	*
HO-2		*
HSP27		*
HSP60	*	*
HSP70	*	*
p21		*
p27		
pp53(S15)	*	
pp53(S46)		
pp53(S392)	*	
pRad17 (S635)		

*$P < 0.05$.

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 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 IκB Kinase (IKK) can also be activated after recruitment to TNF R1, which leads to an inactivation of the inhibitor IκB 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 contrary 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 kd 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 kd 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

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 (Götte, 2003). Taken together the cytokines chosen for the experiments are known to be secreted by the trophoblast during implantation and therewith 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 IITT 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 again. 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 extenuate 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 embryo-maternal 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 IITT 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 TRAIL1/2 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-meditated 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 cIAP-1/2, 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 R1I 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 (Karin and Lin, 2002). Thus the increase of apoptotic proteins during apoptosis may also be regulated by IITT 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 R1I 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 R1I 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

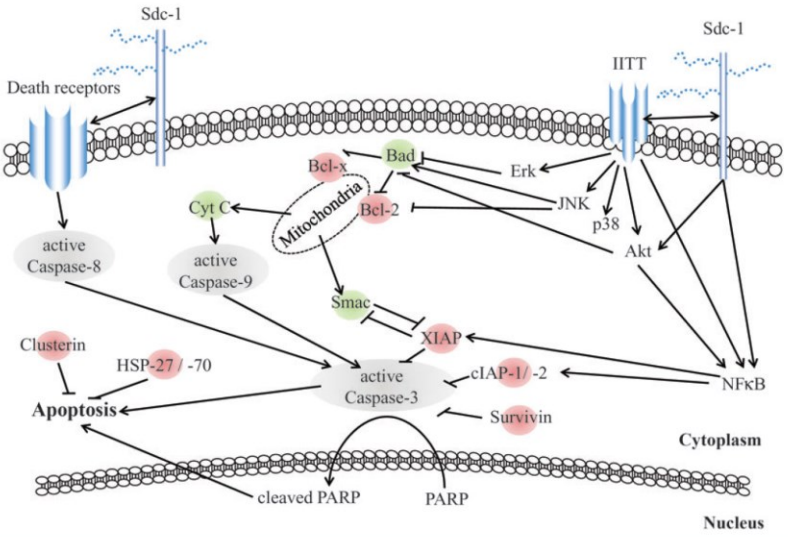

Figure 5 Herein the hypothetical signaling cascade between Sdc-1, Fas/ death receptors, IITT, pro- and anti-apoptotic proteins is depicted. The hypothetical interaction between Sd-1 and death receptors/cytokine receptors is indicated with a double arrow ↔, activating or promoting processes are indicated with an arrow →, blocking or inhibiting processes are indicated with a stop arrow ⟞, pro-apoptotic proteins are labeled green and anti-apoptotic proteins are labeled red.

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 *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 influencing the expression pattern of pro- and anti-apoptotic proteins before and during apoptosis, therewith 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 *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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3.2 Decidualization and Syndecan-1 knock down sensitize endometrial stromal cells to apoptosis induced by embryonic stimuli

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Decidualization and Syndecan-1 Knock Down Sensitize Endometrial Stromal Cells to Apoptosis Induced by Embryonic Stimuli

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Abstract

Human embryo invasion and implantation into the inner wall of the maternal uterus, the endometrium, is the pivotal process for a successful pregnancy. Whereas disruption of the endometrial epithelial layer was already correlated with the programmed cell death, the role of apoptosis of the subjacent endometrial stromal cells during implantation is indistinct. The aim was to clarify whether apoptosis plays a role in the stromal invasion and to characterize if the apoptotic susceptibility of endometrial stromal cells to embryonic stimuli is influenced by decidualization and Syndecan-1. Therefore, the immortalized human endometrial stromal cell line St-T1 was used to first generate a new cell line with a stable Syndecan-1 knock down (KdS1), and second to further decidualize the cells with progesterone. As a replacement for the ethically inapplicable embryo all cells were treated with the embryonic factors and secretion products interleukin-1β, interferon-γ, tumor necrosis factor-α, transforming growth factor-β1 and anti-Fas antibody to mimic the embryo contact. Detection of apoptosis was verified via Caspase ELISAs, PARP cleavage and Annexin V staining. Apoptosis-related proteins were investigated via antibody arrays and underlying signaling pathways were analyzed by Western blot. Non-decidualized endometrial stromal cells showed a resistance towards apoptosis which was rescinded by decidualization and Syndecan-1 knock down independent of decidualization. This was correlated with an altered expression of several pro- and anti-apoptotic proteins and connected to a higher activation of pro-survival Akt in non-differentiated St-T1 as an upstream mediator of apoptotis-related proteins. This study provides insight into the largely elusive process of implantation, proposing an important role for stromal cell apoptosis to successfully establish a pregnancy. The impact of Syndecan-1 in attenuating the apoptotic signal is particularly interesting in the light of an already described influence on pregnancy disorders and therefore might provide a useful clinical tool in the future to prevent pregnancy complications provoked by inadequate implantation.

Introduction

In human, four days after the oocyte was fertilized in the fallopian tube, it reaches the uterus and implants into the inner wall, the endometrium, for further growth and development. Embryo invasion through first endometrial epithelial cells (EECs) and subsequent implantation into the endometrial stromaare crucial steps for a successful pregnancy. This process requires a receptive endometrium, a good quality embryo and a synchronized molecular dialogue between embryo and maternal endometrium [1]. A receptive endometrium is characterized by decidualization of endometrial stromal cells (ESCs) in response to progesterone with morphological changes of the elongated fibroblast-like cells to enlarged, rounded cells [2]. The embryo-maternal dialogue is conducted via secreted cytokines as well as expression of corresponding receptors and co-receptors [3,4]. An alleged important co-receptor for cytokines, which is highly upregulated in the receptive human endometrium is the heparan sulfate proteoglycan Syndecan-1 (Sdc-1) [5]. It is typically present on the cell surface [6], but can also accumulate in the nucleus [7] and exists in the extracellular milieu and body fluids due to proteolytical cleavage from the cell surface as well [8,9]. Hence, the supposed biological functions of Sdc-1 are rather complex and comprise regulation of cell-cell-interaction, cell migration as well as tumorigenesis and consequently attracted interest in the field of obstetrics and gynaecology as well as reproductive medicine in the recent years. Correspondingly, an altered placental Sdc-1 expression was already associated with several pregnancy complications and disorders, which in turn arise from an inadequate implantation [10–12].

The exact cellular mechanisms mediating a proper implantation in human are still not fully understood. Disruption of the endometrial epithelium was intensely investigated and particularly correlated with Fas-mediated apoptosis after binding of the Fas-ligand (FasL)-bearing embryo to the Fas-receptor (FasR) expressing endometrial cell so far [13].

Apoptosis is characterized by fragmentation and engulfing of cell compartments into membrane-covered apoptotic bodies which can be subsequently removed without any immune response or damage of the surrounding cells [14]. It is orchestrated by a cascade of caspases which can be classified in initiator caspases, like Caspase-8 and -9 at the beginning of the pathway and following effector caspases, like Caspase-3 inducing the cellular morphological changes [15]. The Inhibitor of Apoptosis (IAP) family includes different members like XIAP, cIAP-1, -2 and Livin which can bind directly to caspases and thereby inactivate them [16]. Pro-apoptotic molecules like Second mitochondria-derived activator of caspases (SMAC) and High temperature requirement protein A2 (HtrA2) bind IAPs and diminish or even prevent their inhibitory effects on apoptosis on the other side [17]. Cell interactions of pro- and anti-apoptotic molecules are well balanced and altered on demand with a shift to pro-apoptotic proteins leading to the programmed cell death.

Up to date there is little known about a possible role for ESC apoptosis as a consequence to an embryo contact. Evidence from animal studies point out that apoptosis of ESCs plays a major role in the establishment of a pregnancy [18–20] and in contrast to the fast disruption of EECs is regulated in a moderate spatial and temporal fashion [21].

Studies in human are controversial though: mostly a clear role for ESC apoptosis during menstruation is described [22] whereas its role in implantation is not comprehensive. On one hand the presence of scattered apoptotic dESCs in normal human first trimester pregnancy vs. elevated apoptotic dESCs in cases of miscarriage [23] was shown. Furthermore, a study regarding endometriosis, a disease characterized by the presence of endometrial tissue located outside the uterus, described the presence of apopotic ESCs in normal, healthy control individuals throughout the menstrual cycle, which were found to be decreased in patients suffering from endometriosis [24,25]. This is particularly intriguing since on the opposite another publication

depicted a resistance of human ESCs towards apoptosis *in vitro* [26–28]. Therefore, the exact role of ESC apoptosis is ambiguous and still needs to be investigated.

An influence of Sdc-1 on apoptosis was shown before for various cancer cells and revealed a Janus-faced attitude, since in some cancer types, e.g. cervical and endometrial, a low Sdc-1 expression was correlated with high viability and low apoptosis [29,30] whereas in other cancer types, e.g. breast and ovarian, the contrary was observed [31,32].

Therefore, the aim of this study was to investigate the inducibility and regulation of apoptosis in human ESCs induced via embryonic secretion products and surface molecules as a model for embryo contact during implantation to identify the role of apoptosis in conveying a successful pregnancy. In particular, the role of decidualization affecting ESCs regarding their susceptibility to apoptosis should be evaluated. Furthermore, a conceivable influence of Sdc-1 on apoptosis was examined using a stable Sdc-1 knock down (kd) cell line to further clarify its physiological role in human implantation with regard to the regulation of apoptosis.

Materials and Methods

Cell lines, culture conditions and decidualization

The immortalized human endometrial stromal cell line St-T1 was a generous gift from Professor Brosens (University of Warwick, UK), whose group also generated the cell line [33] and initially characterized them for functionality and comparability to primary endometrial stromal cells [34]. St-T1 with a stable and inducible Sdc-1 kd (KdS1) were generated in our laboratory and described before [35]. Cells were maintained at 5% CO_2 and 37°C in a mixture of 75% (v/v) DMEM and 25% (v/v) MCBD 105 supplemented with 10% (v/v) charcoal-stripped fetal calf serum (FCS), 1x penicillin/streptomycin, 40μg/ml gentamycin, 1x sodium pyruvate, 2mM L-glutamine, 1mM non-essential amino acids (all Biowest, Nuaillé, France) and 5μg/ml insulin (Sigma-Aldrich, Steelze, Germany). Cells were decidualized with 0.5mM 8-bromo-cAMP (Biolog, Bremen, Germany) and 1μM medroxyprogesterone 17-acetate (MPA; Sigma-Aldrich) for 72h. Decidualization was proven morphologically via bright-field microscope analysis and biochemically via determination of prolactin (S1 Fig.). Sdc-1 kd of KdS1 was induced applying 1μg/ml tetracycline (tet) for 48h as described before [35].

For the experiments cells were treated with 10ng/ml interleukin (IL)-1β, 10ng/ml interferon (IFN)-γ, 5ng/ml tumor necrosis factor (TNF)-α, 0.5ng/ml transforming growth factor (TGF)-β1 (all Biolegend, San Diego, CA, USA) for 24h alone and in combination (IITT), with 5μg/ml anti-human Fas antibody (ab) clone EOS9.1 (F) (Biolegend) for 24h alone and after 24h pretreatment with IITT (IITT+F) as a replacement for the embryos secretome as established in our laboratory before [36].

Active Caspase-3 measurement

Expression of active Caspase-3 was analyzed applying the Quantikine ELISA (R&D Systems). Non-differentiated St-T1 and KdS1 as well as decidualized St-T1 (dSt-T1) and KdS1 (dKdS1) grown in 24-well plates were treated as described above (n = 3 each). Cell extract preparation and measurement of active Caspase-3 was performed according to the manufacturer's protocol and given as relative amount of active Caspase-3 in ng/ml.

Western blot analysis of poly (ADP-ribose) polymerase (PARP)

Non-differentiated St-T1 and KdS1 as well as dSt-T1 and dKdS1 (n = 3 each) were treated with IITT+F as described above and protein lysates were prepared using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's protocol. Briefly,

30μg protein was separated by 12% SDS-PAGE and transferred to a PVDF membrane (Merck Millipore). The membrane was blocked with 5% non-fat milk and incubated with antibodies against PARP 1:1000 (9542, Cell Signaling Technology) and β-Actin 1:5000 (ab6276, Abcam, Cambridge, UK) at 4°C over night, followed by goat anti-rabbit HRP ab 1:2000 (R&D Systems) for 1h. Signals were detected using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA; USA) and analyzed with the ChemiDoc Imaging System (Bio-Rad) and the corresponding Image Lab software.

Immunfluorescence of Annexin V

Phosphatidylserine-translocation in apoptotic cells was visualized via FITC Annexin V staining of non-differentiated St-T1 and KdS1 as well as dSt-T1 and dKdS1 grown in 35mm dishes (n = 3 each), treated with IITT+F as described above and compared to untreated cells. Cells were washed with PBS containing 2% (v/v) FCS and 0.09% (v/v) sodium azide twice and stained with FITC Annexin V (Bioloegend) diluted 1:20 and 1μg/ml Hoechst 33342 (Sigma-Aldrich) in Annexin V Binding Buffer (Biolegend) for 15min and analyzed with a Zeiss Axiovert 200 microscope and the AxioVision software (Zeiss, Oberkochen, Germany).

Active Caspase-8 and -9 measurements

Non-differentiated St-T1 and KdS1 as well as dSt-T1 and dKdS1 were treated with IITT+F as described above and compared to non-treated controls (n = 5 each). Enzymatic activity of the Caspases-8 and -9 was determined via the corresponding Colorimetric Assay Kit (R&D Systems) according to the manufacturer's manual.

Profiling 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, non-differentiated St-T1 and KdS1 as well as dSt-T1 and dKdS1 (n = 4 each) were first analyzed without a treatment to identify the baseline expression of apoptosis-related proteins and second after treatment with IITT+F as described above. Cell extract preparation for the array with 400μg protein was performed according to the manufacturer's protocol. Dot blots were photographed and analyzed with an Alpha Imager Camera and the corresponding AlphaView Software (Biozym Scientific GmbH, Hessisch Oldendorf, Germany)

FasR analysis

Non-differentiated St-T1 and KdS1 as well as dSt-T1 and dKdS1 were treated with IITT 24h and compared to non-treated controls (n = 5 each). Induction of FasR after treatment with IITT was investigated by real time PCR using the 2^-ΔΔCt method [37]. Primers for FasR were for 5'-GGACCCTCCTACCTCTGGTTCTTA-3' and rev 5'-TTCACCTGGAGGACAGGG CTT-3' (annealing temperature 50°C) and were normalized to the housekeeping gene β-Actin; primers were 5'-CGGGACCTGACTGACTACC-3' and rev 5'-AGGAAGGCTGGAAGAGT GC-3' (annealing temperature 50°C).

Western blot analysis of Akt, JNK, p65 and IκBα

Non-differentiated St-T1 and KdS1 as well as dSt-T1 and dKdS1 were treated with IITT for 15min, F for 15min as well as IITT 24h + F for 15min and compared to untreated controls (n = 6 each). Experiments to determine a time dependent expression were performed in our laboratory before. Protein lysates were prepared with Cell Lysis Buffer (Cell Signaling

Technology) according to the manufacturer`s protocol. 30µg protein was separated by 12% SDS-PAGE and transferred to a PVDF membrane (Merck Millipore). The membrane was blocked with 5% non-fat milk and incubated with antibodies against Akt (9542), phosphorylated (p)Akt (4060), JNK (9258), pJNK (4668), p65 (8242), pp65 (3039) IκBα (4814) all 1:1000 (all Cell Signaling Technology) and β-Actin (ab6276, Abcam) at 4°C over night, followed by goat anti-rabbit or goat anti-mouse HRP ab 1:2000 (R&D Systems) for 1h. Signals were detected using Clarity Western ECL Substrate (Bio-Rad) and analyzed with the ChemiDoc Imaging System (Bio-Rad) and the corresponding Image Lab software.

Statistical analysis

Two groups each were analyzed applying unpaired 2-tailed Mann-Whitney-U-Tests, and ANOVAs with Bonferroni post hoc tests were used for multiple comparisons by using SPSS 22 (SPSS, Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant.

Results

Quantification of active Caspase-3 in ESCs treated with embryonic stimuli

First, induction of apoptosis was quantified by investigating the total amount of active Caspase-3 after treatment with IITT and F separately or in combination (Fig. 1). Non-

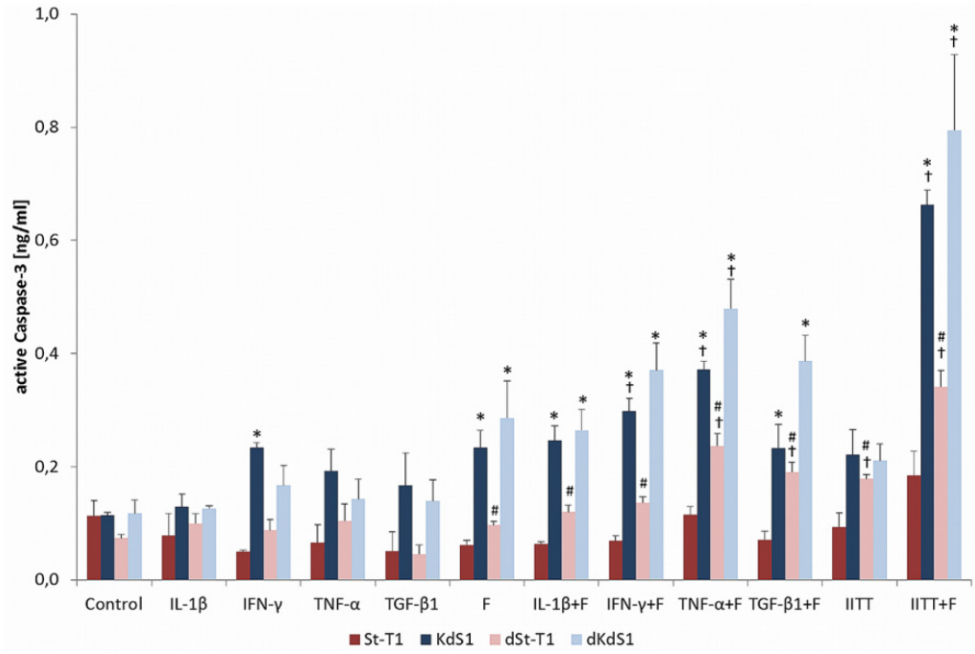

Fig 1. Quantification of active Caspase-3 in ESCs treated with embryonic stimuli. Non-differentiated (St-T1, red bar; KdS1, blue bar) and decidualized (dSt-T1, bright red bar; dKdS1, bright blue bar) ESCs were treated with IITT and F for 24h individually or in combination as indicated and the amount of active Caspase-3 was analyzed in ng/ml and displayed as mean±SEM of n = 3 independent experiments; *$p<0.05$ Sdc-1 wildtype vs. Sdc-1 kd cells, #$p<0.05$ non-differentiated vs. decidualized cells, †$p<0.05$ untreated controls vs. treated cells.

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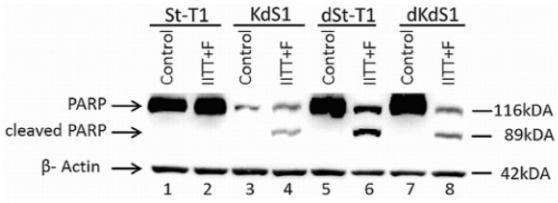

Fig 2. PARP-cleavage upon treatment with embryonic stimuli. Representative Western Blot analysis of PARP cleavage (full length PARP 116kDa; cleaved PARP 89 kDa) in non-differentiated (St-T1, lanes 1+2; KdS1, lanes 3+4) and decidualized (dSt-T1, lanes 5+6; dKdS1, lanes 7+8) ESCs after treatment with IITT+F vs. untreated controls, n = 3; β-Actin (42kDa) served as a loading control.

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differentiated St-T1 showed no significant increase of active Caspase-3 compared to untreated control and consequently no induction of apoptosis after all tested treatments. In contrast KdS1 revealed a significant increase of active Caspase-3 compared to control after treatment with combination of IFN-γ+F as well as TGF-β1+F and combination of IITT+F. In addition dSt-T1 induced apoptosis after treatment with TNF-α+F, TGF-β1+F, IITT and IITT+F whereas dKdS1 displayed apoptosis induction after treatment with TNF-α+F and likewise IITT+F. The latter incubation condition led to the highest increase of active Caspase-3 in all cells except non-differentiated St-T1 with a significant raise due to the Sdc-1 kd (St-T1 vs. KdS1 and dSt-T1 vs. dKdS1) and according to decidualization (St-T1 vs. dS-T1 and KdS1 vs. dKdS1).

PARP-cleavage upon treatment with embryonic stimuli

After treatment of cells with IITT+F cleavage of PARP vs. untreated controls as the main downstream target of active Caspase-3 was shown via Western Blot analysis in KdS1, dSt-T1 and dKdS1 (Fig. 2), whereas no cleavage was seen in St-T1.

Loss of membrane asymmetry after IITT+F treatment

The loss of membrane asymmetry with a phosphatidylserine-translocation from the inner to the outer cell membrane as a consequence of apoptosis was investigated by staining of cells with Annexin V after treatment with IITT+F. KdS1 (Fig. 3F), dSt-T1 (Fig. 3G) and dKdS1 (Fig. 3H) showed a green staining whereas none was detectable in the controls (Fig. 3A–3D) and IITT+F treated St-T1 (Fig. 3E). In the latter only a light green staining was observed possibly indicating spontaneous apoptosis. The bright green spots without a cell shape display artefacts.

Investigation of the extrinsic and intrinsic apoptosis pathway

To study the activation of pro-Caspase-8 and -9, incubation with IITT+F was chosen. Non-differentiated KdS1, dSt-T1 and dKdS1 showed a significant induction of Caspase-8 (Fig. 4A) and -9 (Fig. 4B) after treatment when compared to untreated controls which was assigned 1. No Caspase-8 induction was found for treated St-T1 vs. untreated control and for Caspase-9 only a slight but not statistically significant induction was observed. Within the group of treated cells (St-T1, KdS1, dSt-T1, dKdS1), only Caspase-8 induction was significantly higher in KdS1 compared to St-T1.

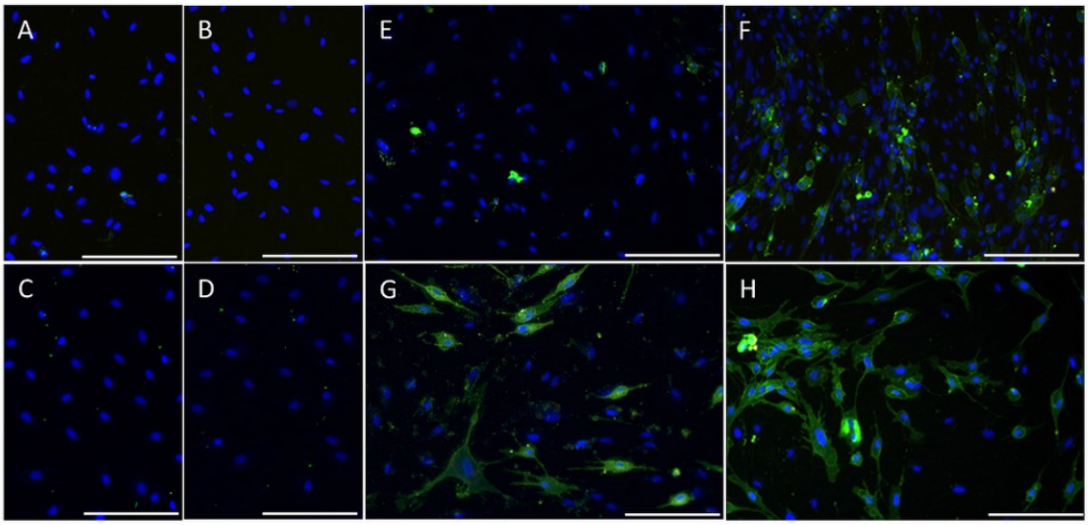

Fig 3. Loss of membrane asymmetry after IITT+F treatment. Non-differentiated (St-T1, KdS1) and decidualized (dSt-T1, dKdS1) ESCs were treatmed with IITT+F and loss of membrane asymmetry was visualized with Annexin V FITC staining (green), n = 3; (A)–(D) untreated controls: (A) St-T1, (B) KdS1, (C) dSt-T1, (D) dKdS1. (E)-(H) IITT+F treated: (E) St-T1, (F) KdS1, (G) dSt-T1, (H) dKdS1; blue nuclei are stained with Hoechst 33342. Scale bars indicate 100μm.

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Expression of apoptosis-related proteins before and after IITT+F treatment

The analysis of apoptosis-related proteins in untreated cells as a baseline expression (Fig. 5A) demonstrated a statistically significant increase of pro-apoptotic Bad in accordance to decidualization and additive to the Sdc-1 kd. The death receptor FasR was also increased in both decidualized cell types dSt-T1 and dKdS1, whereas anti-apoptotic Livin was decreased. Pro-

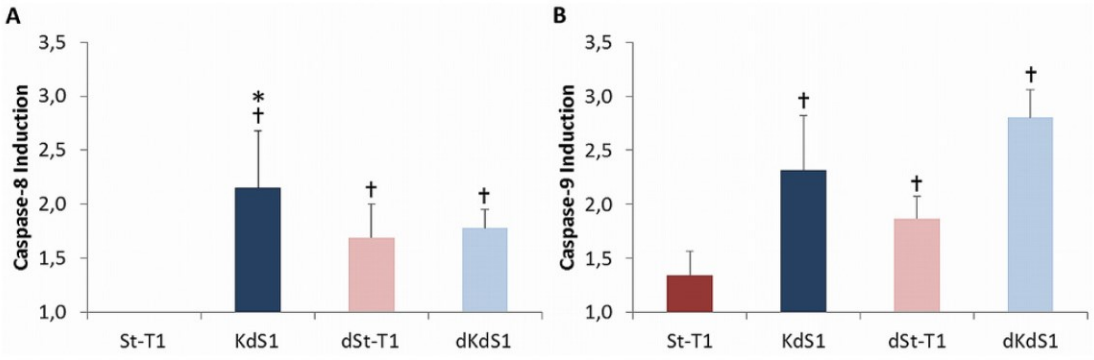

Fig 4. Investigation of the extrinsic and intrinsic apoptosis pathway after IITT+F treatment. Analysis of Caspase-8 and -9 activation of treated cells vs. untreated controls in non-differentiated (St-T1, red bar; KdS1, blue bar) and decidualized dSt-T1, bright red bar; dKdS1, bright blue bar) ESCs after treatment with IITT+F. Untreated controls were assigned being 1 and enzymatic activity of caspases after treatment was determined as fold induction vs. controls and given as mean±SEM of n = 3 independent experiments, *$p<0.05$ Sdc-1 wildtype vs. Sdc-1 kd cells, †$p<0.05$ untreated controls vs. treated.

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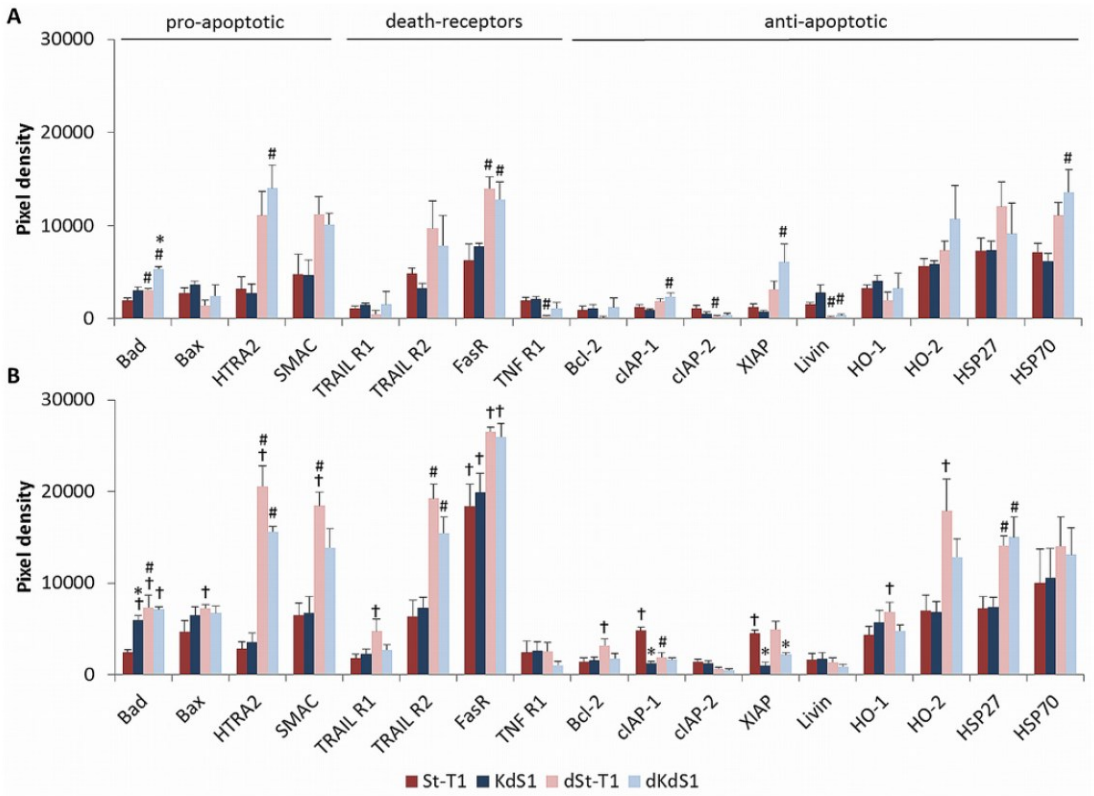

Fig 5. Expression of apoptosis-related proteins before and after IITT+F treatment. Antibody array analysis of apoptosis-related proteins in ESCs of (A) untreated, non-differentiated (St-T1, red bar; KdS1, blue bar) and decidualized (dSt-T1, bright red bar; dKdS1, bright blue bar) ESCs, n = 4 ± SEM, *p<0.05 Sdc-1 wildtype vs. Sdc-1 kd cells, #p<0.05 non-differentiated vs. decidualized cells. (B) Antibody Array with protein from IITT+F treated, non-differentiated (St-T1, red bar; KdS1, blue bar) and decidualized (dSt-T1, bright red bar; dKdS1, bright blue bar) ESCs. Pixel density is given as mean±SEM of n = 4 independent experiments, *p<0.05 Sdc-1 wildtype vs. Sdc-1 kd cells, #p<0.05 non-differentiated vs. decidualized cells, †p<0.05 untreated vs. IITT +F treated.

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apoptotic HTRA2 and anti-apoptotic cIAP-1, XIAP and heat shock protein (HSP) 70 were increased in dKdS1 compared to non-differentiated KdS1, while cIAP-2 and TNF R1 were decreased in dSt-T1 compared to St-T1.

Fig. 5B shows that treatment with IITT+F led to a significant upregulation of several proteins: pro-apoptotic Bad in KdS1, dSt-T1 and dKdS1; Bax, HTRA2, SMAC, TNF-related apoptosis-inducing ligand receptor (TRAIL R1) and anti-apoptotic Bcl-2, heme oxygenase (HO)-1 and -2 in dSt-T1. FasR was significantly upregulated in all cell types, whereas anti-apoptotic cIAP-1 and XIAP increased in St-T1. As a consequence Bad was significantly higher in accordance to the Sdc-1 kd in KdS1 whereas cIAP-1 and XIAP were significantly lower. Bad, HTRA2, SMAC, TRAIL R2 and HSP27 were significantly higher dependent on decidualization, while cIAP-1 was downregulated in dSt-T1 compared to St-T1.

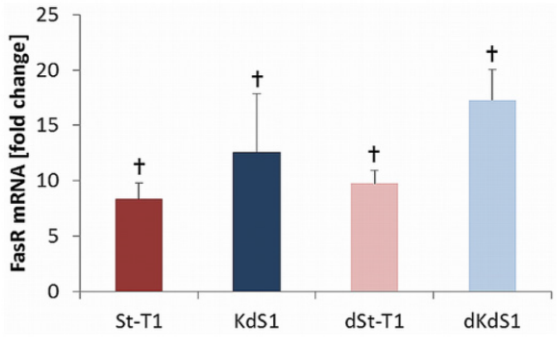

Fig 6. Induction of FasR expression after IITT treatment. Fold change of FasR mRNA in non-differentiated (St-T1, red bar; KdS1, blue bar) and decidualized (dSt-T1, bright red bar; dKdS1, bright blue bar) ESCs after treatment with IITT 24h. $2^{-\Delta\Delta Ct}$ is are given as mean±SEM of n = 5 independent experiments, †$p<0.05$ untreated vs. IITT treated.

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Induction of FasR expression after IITT

FasR protein was significantly upregulated in all investigated cell types after IITT+F treatment (Fig. 5B). *Real time* PCR analysis showed FasR mRNA was already upregulated after single treatment with IITT for 24h without F (Fig. 6).

Analysis of apoptosis-related signaling pathways

Short time incubation treatments for 15min were performed to analyze a possible activation of the cell signaling proteins Akt, nuclear factor (NF)κB members p65 and inhibitor of kappa B (IκBα) as well as mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK). Fig. 7 illustrates that Akt was constitutively active in non-differentiated St-T1. Interestingly, the Sdc-1 kd in KdS1 or decidualization in dSt-T1 led to a decrease of pAkt. Furthermore a strong additive decrease of pAkt in dKdS1 was observed. Correspondingly, the pixel density analysis revealed a significant decrease compared to St-T1 in the controls and after short treatment with IITT 15min (Fig. 7B).

The NFκB member pp65 (Fig. 8A and 8B) as an indirect downstream target of pAkt was not upregulated in non-differentiated St-T1, whereas pp65 rather significantly increased in response to decidualization in dSt-T1 and dKdS1 vs. non-differentiated cells. Furthermore, a strong induction after IITT treatment for 15min led to a significant increase of pp65 compared to control. Accordingly, the corresponding inhibitor IκBα (Fig. 8C and 8D) was downregulated after IITT treatment for 15min, but no differences between non-differentiated and decidualized stromal cells were observed.

Finally Fig. 9A and 9B depict that pJNK as a common mediator of apoptosis is significantly increased in KdS1, dSt-T1 and dKdS1 after treatment with IITT for 15min, whereas no statistically significant increase was observed for St-T1 treated with IITT for 15min.

Discussion

The attachment of an embryo to the maternal endometrial epithelium and its consequent invasion in first the endometrial epithelium and second the endometrial stroma is an indispensable prerequisite for a successful pregnancy. Although it is described that cell death of EECs is the

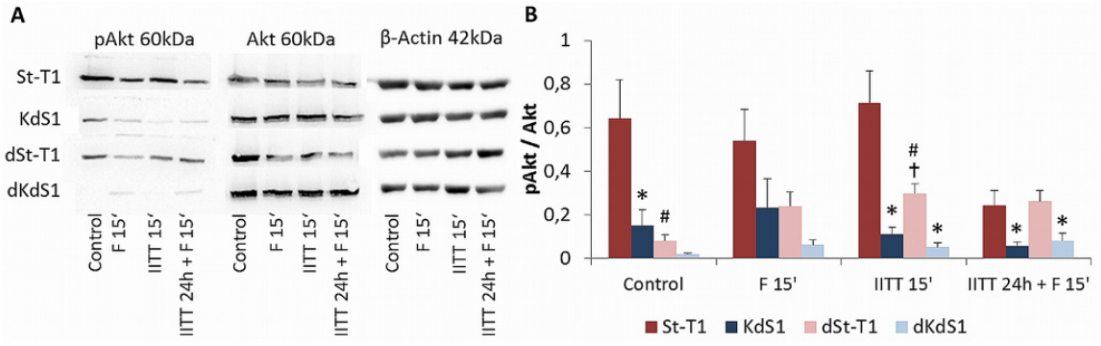

Fig 7. Activation of the pro-survival protein Akt after IITT and F treatment. Western blot analysis of pAkt and Akt in non-differentiated (St-T1, first line; KdS1, second line) and decidualized (dSt-T1, third line; dKdS1, fourth line) ESCs after treatments with F 15min, IITT 15min and IITT 24h + F 15min vs. untreated controls. (A) Representative blot of pAkt (60kDa), Akt (60kDa) and β-Actin (42kDa) as loading control. (B) Pixel densitiy evaluation of pAkt normalized to Akt is given as mean±SEM of n = 6 independent experiments, *$p<0.05$ wildtype vs. Sdc-1 kd cells, #$p<0.05$ undifferentiated vs. decidualized cells, †$p<0.05$ untreated control vs. treated.

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modus operandi how the embryos way into the stroma is facilitated [13], little is known about the role of apoptosis within ESCs to achieve the appropriate invasion depth for the implanting embryo. If anything, there are rather controversial studies describing both presence and resistance of ESCs towards apoptosis [26,38].

Hence, in the present study the competence of embryonic stimuli (IITT+F) to mimic the situation of embryo implantation regarding their apoptotic competence in human ESCs was fathomed closer. The aim was to achieve detailed information about the possible role for ESC apoptosis during the embryo implantation process. Furthermore, the influence of Sdc-1 on the apoptotic signal and expression of apoptosis-related proteins was investigated using a stable Sdc-1 kd cell line. Since we have shown before that Sdc-1 modulates apoptosis in EECs revealing an increase of apoptotic sensitivity towards IITT+F in connection with a downregulation of anti-apoptotic proteins and upregulation of the death receptors FasR and TRAIL R 2 by Sdc-1 kd [36]. This study is limited to *in vitro* experiments with immortalized cell lines, to achieve reproducible insights of Sdc-1's influences using a stable knock down. Furthermore, treatment of cells with 4 cytokines (IITT) and anti-Fas ab (F) was applied as a replacement to mimic embryo contact in the absence of a more suitable equivalent, as described previously [36]. In detail IL-1β was chosen because it is highly secreted by human embryos *in vitro* [39,40]. Furthermore IFN-γ is also expressed by the early embryo and uterine immune cells which rapidly expand in early pregnancy [41]. TNF-α and the corresponding receptors are expressed in the endometrium, placenta and the fetus during pregnancy [42]. TGF-β1 is expressed in endometrial stromal cells [43], in the early embryo [44] and in the placenta [45].

Studies in endometrial cancer showed that upregulated Sdc-1 promotes the viability of the cells via activation of NFκB [29] whereas silencing of Sdc-1 induced apoptosis correlated with a decrease in the activation of MAPK Erk and Akt [46]. Additionally, Sdc-1 kd was shown to enhance the sensitivity to TRAIL-induced apoptosis [47]. Hence, in spite of the contrary literature, an influence of Sdc-1 kd in ESC apoptosis by modulation of MAPK, Akt and NFκB or death receptor signaling is conceivable although up to date the knowledge about the exact mechanism is very limited.

Clinical studies correlated an altered Sdc-1 expression with the risk of pregnancy diseases like preeclampsia, fetal growth restriction and preterm delivery, pregnancy complications

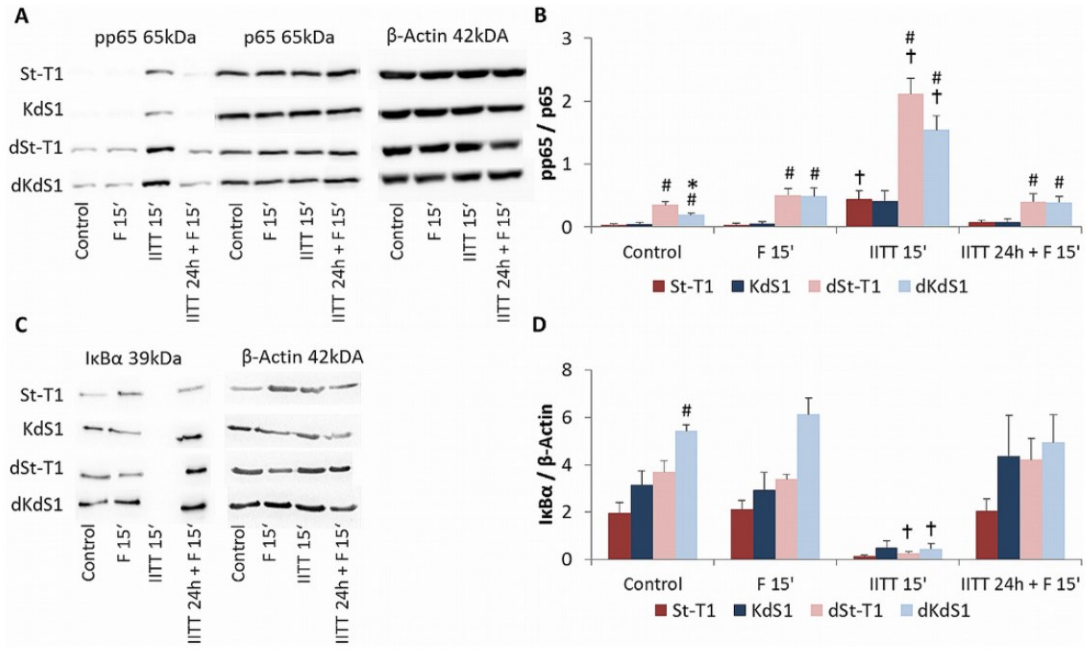

Fig 8. Activation of the pro-survival NFκB pathway after IITT and F treatment. Western blot analysis of NFκB members p65 and IκBα in non-differentiated (St-T1, first line; KdS1, second line) and decidualized (dSt-T1, third line; dKdS1, fourth line) ESCs after treatments with F 15min, IITT 15min and IITT 24h + F 15min vs. untreated controls. (A) Representative blot of pp65 (65kDa), p65 (65kDa) and β-Actin (42kDa) as loading control. (B) Pixel densitiy evaluation of pp65 normalized to p65 is given as mean±SEM of n = 6 independent experiments, *p<0.05 wildtype vs. Sdc-1 kd cells, #p<0.05 undifferentiated vs. decidualized cells, †p<0.05 untreated control vs. treated. (C) Representative blot of IκBα (39kDa) and β-Actin (42kDa) as loading control. (D) Pixel densitiy evaluation of IκBα normalized to β-Actin is given as mean±SEM of n = 6 independent experiments, *p<0.05 wildtype vs. Sdc-1 kd cells, #p<0.05 undifferentiated vs. decidualized cells, †p<0.05 untreated control vs. treated.

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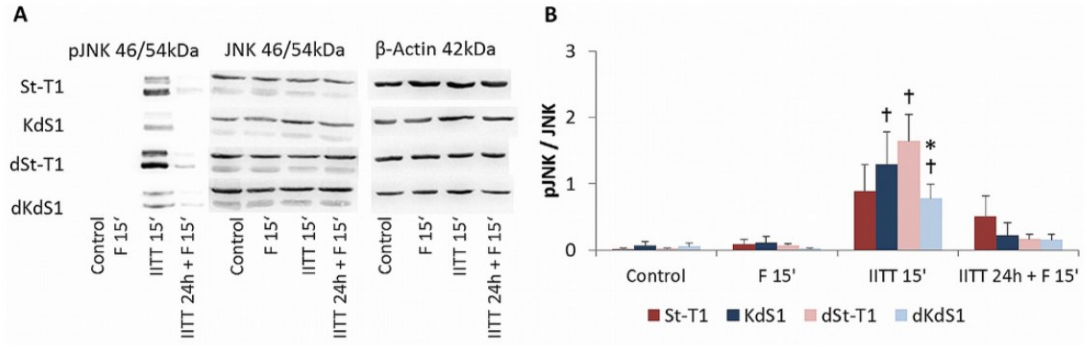

Fig 9. Activation of the pro-apoptotic JNK pathway after IITT and F treatment. Western blot analysis of pJNK and JNK in non-differentiated (St-T1, first line; KdS1, second line) and decidualized (dSt-T1, third line; dKdS1, fourth line) ESCs after treatments with F 15min, IITT 15min and IITT 24h + F 15min vs. untreated controls. (A) Representative blot of pJNK (46/54kDa), JNK (46/54kDa) and β-Actin (42kDa) as loading control. (b) Pixel densitiy evaluation of pJNK normalized to JNK is given as mean±SEM of n = 6 independent experiments, *p<0.05 wildtype vs. Sdc-1 kd cells, †p<0.05 untreated control vs. treated.

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which in turn are allocated most likely due to insufficient invasion [10–12,48]. Therefore, we hypothesized that Sdc-1 facilitates a proper implantation via regulating maternal cell apoptosis as a reaction to a contact with the embryo and its secretome.

The ESCs used in this study were able to be decidualized *in vitro* in the presence of cAMP and MPA. Consequently, four different cell conditions (non-differentiated with and without Sdc-1 kd and decidualized with and without Sdc-1 kd) were generated for all experiments. Analyzing the inducibility of apoptosis in reaction to treatment with IITT+F, the non-differentiated St-T1 revealed a resistance towards apoptosis under all tested incubation conditions. In more detail, no significant increase of Caspase-3 activation after treatment with IITT+F was detectable in St-T1 and consequently no PARP cleavage as the main downstream target of active Caspase-3 and a marker for cells undergoing irreversible apoptosis [49]. Additionally, there was no loss of membrane asymmetry illustrated via Annexin V staining emphasizing the St-T1 resistance towards apoptosis. Decidualization though seemed to sensitize the stromal cells for IITT+F mediated apoptosis. Interestingly, the Sdc-1 kd independent of decidualization rendered the cells for apoptosis as well. Subsequently, decidualized stromal cells with Sdc-1 kd displayed an even greater magnitude of apoptosis induction, leading to the highest active Caspase-3 activation in dKdS1. In accordance with the Caspase-3 activation, cleavage of PARP and Annexin V staining were also observed in KdS1, dSt-T1 and dKdS1 after treatment with IITT+F.

The apoptotic process can be started by two major ways: extrinsic via death receptors, like FasR or TRAIL R, mediated by Caspase-8 [50] or intrinsic by permeabilization of mitochondria and activation of Caspase-9. Both pathways administrate the activation of Caspase-3. To decipher whether the extrinsic or intrinsic apoptotic pathway is initiated via IITT+F treatment, the induction of corresponding caspases was analyzed, indicating an equivalent activation of both Caspase-8 and -9 after treatment with IITT+F in KdS1, dSt-T1 and dKdS1. Therefore, we assumed that Caspase-8 is activated as a consequence of F treatment which binds and activates correspondingly FasR, whereas Caspase-9 activation is possibly a secondary effect after Caspase-8 induced cleavage of the protein BID (BH3 interacting domain death agonist) which as a consequence initiates mitochondrial damage [51].

To find out, which apoptosis-related proteins are involved in regulating the divergence in apoptotic susceptibility, antibody arrays were conducted. The basal protein expression without induction of apoptosis revealed an increase of pro-apoptotic Bad in both decidualized cell types dSt-T1 and dKdS1 compared to the non-differentiated cells and therewith suggests a higher apoptotic susceptibility in decidualized cells even without further treatment. Additionally, pro-apoptotic Bad was further upregulated in dKdS1 compared to dSt-T1, which might result from an additive effect of decidualization and Sdc-1 kd. FasR also increased in accordance to decidualization, whilst anti-apoptotic Livin decreased due to decidualization in dSt-T1 and dKdS1. Data of upregulated stromal FasR expression in secretory compared to proliferative phase endometrium proposed a preparation of the stromal cells for the arriving FasL-bearing embryo [52].

However, IITT+F treatment provoked an increase of abundant proteins in all different cell types in varying degrees. Bad was upregulated in KdS1, dSt-T1 and dKdS1 and significantly higher compared to St-T1, whereas anti-apoptotic cIAP-1 and XIAP were significantly increased in St-T1. CIAP-1 and -2 and XIAP are members of the IAP family, which serve as suppressors of Fas-mediated apoptosis through direct Caspase-3 inhibition and modulation of the transcription factor NFκB [53]. Furthermore, XIAP is known to mediate first trimester trophoblast resistance to Fas-mediated apoptosis regardless of expressing both FasR and FasL [54], which must be kept in mind since dESCs also express both FasR and FasL [52]. Interestingly, FasR was increased in all cell types treated with IITT+F and supplemental experiments

revealed, that FasR was already induced by single treatment with IITT on mRNA level, which might sensitize the cells to Fas-mediated apoptosis and showed a possibly important role for embryo-maternal FasL/FasR interaction in ESCs, as we have already described it for EECs before [36]. FasR was upregulated even in apoptosis resistant St-T1, but obviously there was no transfer of the apoptotic signal from the receptor to Caspase-3 since it was unchanged, which probably resulted from the influence of upregulated anti-apoptotic proteins specified before. It was described in the literature that pre-treatment with TNF-α/IFN-γ sensitized primary cultured ESCs to Fas-mediated apoptosis, which were resistant to single Fas-mediated apoptosis before. This was accompanied by an increase of FasR as well as an induction of Caspase-3, -8 and -9. In contrast to our findings apoptosis was induced in both non-differentiated and decidualized ESCs and there was no difference in FasR expression between ESCs and dESCs before and after TNF-α and IFN-γ treatment [28]. This discrepancy might result from the usage of different cell types, concentrations of cytokines and Fas Ab, as well as different Fas Ab clones and different detection methods applied. In our experiments another death-receptor, TRAIL R2, was significantly upregulated in IITT+F treated and decidualized cells, which indicates an additional role for the TRAIL/TRAIL R system besides FasL/FasR in implantation correlated apoptosis. It is already described that even the ligand TRAIL is upregulated upon *in vitro* decidualization [55] and expressed in dESCs in first trimester placental biopsies combined with the corresponding receptors TRAIL R1 and 2 [56]. To gain a deeper insight into signaling pathways regulating the observed apoptosis, Western blot analysis was performed to identify signaling molecules with a supposable impact on regulating the apoptotic signal in ESCs. Short time incubation with IITT, F alone and in combination was chosen, because the investigated signaling pathways are regulated quickly within 15min. In St-T1 the protein Akt was shown to be constitutively phosphorylated. A significant decrease due to the Sdc-1 kd and decidualization in the untreated controls and after IITT treatment was observed. Akt is a common mediator of viability, inhibiting the expression of pro-apoptotic proteins like Bad and promoting expression of anti-apoptotic protein like IAPs. Hence, the significant increase of anti-apoptotic proteins in St-T1, as well as the increase of pro-apoptotic Bad in KdS1, dSt-T1 und dKdS1 detected in the antibody arrays might be a consequence of increased Akt-activation in St-T1 as seen in this study. An influence of Sdc-1 on Akt-signaling was already described for endometrial cancer cells as aforementioned. Furthermore, it is known that Sdc-1 promotes HGF/Met signaling, resulting in enhanced activation of signaling pathways involved in the control of cell proliferation und survival, like RAS/MAPK and PI3K/Akt [57]. It was also described, that primary cultured ESCs constitutively secreted HGF *in vitro* [58]. Therefore, increased Akt activation in St-T1 could be regulated in an autocrine manner via secreted HGF, activation of Met and consequently Akt orchestrated by Sdc-1. Consequently, Sdc-1 missing KdS1 display a decreased pAkt level leading to higher apoptotic susceptibility as observed in this study. Decrease of pAkt upon decidualization was already described indirectly for primary ESCs [59], because it was shown that PTEN, functioning as an inhibitor of the PI3K/Akt pathway increased in dESCs during the late secretory phase [60]. Forkhead box protein O1 (FoxO1a) is a transcription factor in the cell nucleus, which participates in the induction of apoptosis [61]. Moreover, phosphorylation of FoxO1a via pAkt leads to an exclusion of FoxO from the nucleus to the cytoplasm, which is associated with reduced transcriptional activity. It is described that unphosphorylated FoxO1a accumulates in the nuclei of dESCs *in vivo* and this is correlated with a suppression of PI3K/Akt pathway upon decidualization [62]. Thus, decreased pAkt in dSt-T1 compared to St-T1 might also be a consequence to decidualization in our study.

Subsequently, two members of the NFκB protein family, p65 and IκBα, as indirect targets of pAkt were analyzed. A significant increase of pp65 in all decidualized cells with a strong induction after IITT treatment was detected but no increase in undifferentiated St-T1 as a

consequence of increased Akt activation. Therefore, it seems as if Akt developed its anti-apoptotic properties via modifying other pro- and anti-apoptotic proteins besides NFκB. Consistent with the increase of pp65, the p65 inhibitor IκBα was degraded after IITT treatment, even though the difference between non-differentiated and decidualized cells as found for pp65 was not statistical significant for IκBα. A possible reason could be that only one of four members of the IκB family was studied.

Finally, the MAPKs JNK1/2 were investigated and again a significant increase in KdS1, dSt-T1 and dKdS1 after IITT treatment was seen compared to the untreated controls. In contrast, pJNK was not increased in St-T1. This indicates that apoptosis in KdS1, dSt-T1 and dKdS1 is potentially mediated via pJNK.

Taken together, *in vitro* cultured ESCs treated with IITT+F showed a resistance toward apoptosis, mediated via constitutive active Akt. Decidualization of these cells abrogated the resistance in connection with a decrease of pAkt and correspondingly an altered expression of Akt-regulated pro- and anti-apoptotic proteins. Furthermore, the Sdc-1 kd sensitized St-T1 to apoptosis independent of decidualization and in all apoptosis-sensitive cells the highest induction of Caspase-3 was measured after a combined treatment of IITT+F.

Hypothetical mechanisms being subject to the influence of Sdc-1 on ESC apoptosis might be regulating signaling pathways like MAPK or PI3K via the mediator Akt and the corresponding pro- and anti-apoptotic proteins as well as the transcription factor FoxO1a indirectly by modulation of cytokine and death receptor binding to their corresponding ligands. Additionally, a direct influence on the extrinsic death-receptor mediated pathway or on the Akt-pathway itself is conceivable.

In summary, decidualization which is necessary for proper embryo implantation provides the opportunity for the endometrial stromal cell line St-T1 to induce apoptosis in reaction to embryonic stimuli *in vitro*, which provides an indication for ESC apoptosis as an important process during implantation *in vivo*. Even if the exact Sdc-1 influence on apoptosis induction is not fully understood so far the overall findings of this study indicate that Sdc-1 may act as a modulator of ESC apoptosis and probably invasion depth as a crucial factor for successful pregnancy. Hence, the important role of Sdc-1 in influencing implantation which has been discussed extensively already and the background that the pathophysiology of preeclampsia and fetal growth restriction is correlated with an altered Sdc-1 expression on the one hand [12,48,63] and increased placental cell apoptosis on the other [64,65] supports our thesis that Sdc-1 exerts its influence on implantation via regulating ESC apoptosis.

Supporting Information

S1 Fig. Proof of decidualization markers. Representative bright-field microscope analysis of cell morphology before and after decidualization *in vitro*: (A) non-differentiated St-T1, (B) dSt-T1 after decidualization via MPA and cAMP treatment for 72h; scale bar indicates 100μm. (C) representative PCR gel for the housekeeping gene HMBS (64bp) in St-T1 (lane 1+2), KdS1 (lane 3+4), dSt-T1 (lane 5+6) and dKdS1 (lane 7+8). (D) representative PCR gel for the decidualization marker PRL (247bp) in dSt-T1 (lane 5+6) and dKdS1 (lane 7+8) vs. non differentiated St-T1 (lane 1+2) and KdS1 (lane 3+4); M = marker and (-) negative control without template. (E) quantification of secreted PRL via ELISA in in non-differentiated (St-T1, red bar; KdS1, blue bar) and decidualized (dSt-T1, bright red bar; dKdS1, bright blue bar) ESCs. Values are given as mean±SEM of n = 3 replicates.
(TIF)

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Author Contributions

Conceived and designed the experiments: SJB DMBB AH. Performed the experiments: SJB. Analyzed the data: SJB DMBB AH. Contributed reagents/materials/analysis tools: TF JK AH. Wrote the paper: SJB DMBB TF JK AH.

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3.3 The role of apoptosis in human embryo implantation

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The role of apoptosis in human embryo implantation

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ABSTRACT

The process of embryo attachment and invasion through the endometrial epithelial cells and subsequent implantation into the decidualized endometrial stroma is the groundbreaking step for the establishment of a successful pregnancy. Necessary prerequisites are a receptive endometrium, a good-quality embryo and a well-orchestrated molecular dialog between embryo and maternal endometrium. The embryo–maternal dialog is conducted *via* a wide scope of factors, including secreted cytokines, chemokines, and growth factors in addition to the expression of corresponding receptors and co-receptors. Several embryonic proteins, including the aforementioned, are involved in the process of apoptosis, which necessarily needs to take place at the maternal endometrium to allow the embryo to invade. The endometrial epithelium is thereby disintegrated completely within a particular area, whereas the endometrial stroma seems to require a more depth-limited apoptosis. As of today, the exact mechanisms and factors mediating the apoptotic process involved in those apparently differently regulated incidents are not fully understood, particularly with regard to stromal cell apoptosis. There is evidence though, that cytokines and their respective receptors play a major role. A suggested important co-receptor for cytokines, which is highly upregulated in the receptive human endometrium, is the heparan sulfate proteoglycan syndecan-1. It is present on the cell surface and involved in the regulation of cell–cell-interaction, cell binding, cell signaling and cytoskeletal organization and therefore represents a possible mediator of apoptosis regulation in human endometrium. Herein, the literature on endometrial epithelial and stromal apoptosis in general, and in light of the influence of syndecan-1, is reviewed.

1. Introduction

1.1. Embryonic implantation

Human endometrium undergoes regular cyclic changes throughout each menstrual cycle scheduling for embryo implantation, and in its absence, for menstruation. Both processes are accompanied by cellular mitosis, differentiation, and apoptosis. Embryo implantation into a receptive endometrium is the pivotal process in early pregnancy influencing the course of pregnancy tremendously. The embryo–maternal dialog, which is pivotal for successful implantation and subsequent pregnancy, is conducted *via* a broad spectrum of factors including secreted cytokines and chemokines in addition to the expression of their corresponding receptors and co-receptors. After leaving its protective zona pellucida, the embryonic trophoblast facilitates contact with the endometrium and starts penetration of the endometrial epithelium leading to complete apoptosis of the epithelial layer in a locally determined fashion, followed by invasion into the endometrial stroma and the inner third of the myometrium, which seems to

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be rather distinctly regulated in space (Cohen et al., 2010). Disturbance of this tightly regulated procedure can lead to inadequate implantation, which in turn enables the occurrence of several diseases such as intrauterine growth restriction (IUGR), HELLP syndrome, and/or preeclampsia (Pijnenborg et al., 2008). It is discussed, though, whether inadequate invasion might just be a trigger inducing the disease, if combined with further preexisting conditions, leading to an incomplete vascular transformation, reduced uteroplacental blood flow, and the secretion of inducers of endothelial cell activation and vascular damage into the maternal circulation, which ultimately causes hypertension and proteinuria (Borzychowski et al., 2006). Nevertheless, insufficient invasion seems to play a role in the development of serious pregnancy diseases either as a trigger or as a self-contained cause. Since embryo implantation is a multifactorial and constitutive event, an early disturbance at the level of embryo invasion may ultimately derail implantation, although the presence of a good-quality embryo and an otherwise receptive endometrium. In addition, from an immunological point of view it is very interesting that apoptosis at the embryo–maternal interface, which in terms of Matzinger's "Danger Model of Immunity" is a "danger" signal and therefore should lead to a danger-triggered conceptus rejection, does not provoke this pregnancy-limiting step and therefore may belong to an inert fetal strategy that explains the paradox of the fetal allograft in humans (Matzinger, 2002). Therefore, it is important to understand the mechanisms involved in appropriately guided apoptosis in humans.

In recent years, the interest in endometrial apoptosis as a mandatory prerequisite for successful pregnancy has yielded more and more interest. As the process seems to be very diverse in endometrial epithelial cells (EECs) compared with endometrial stromal cells (ESCs) those entities will be reviewed separately in the following paragraphs. Additionally, animal studies will be incorporated into the discussion as limited studies in human endometrial stromal cell apoptosis exist. Furthermore, the cytokine receptor syndecan-1 (Sdc-1), which was described to play a role in the apoptosis of tumor cells and whose ligands are also known to be involved in the implantation process will be reviewed herein as an interesting possible regulator of apoptosis in human endometrium, influencing the implantation process.

1.2. Apoptosis

The term "apoptosis" describing an active, strict, and complex regulated process of eliminating single cells without the response of inflammation and damage to the surrounding cells was introduced in 1972, documenting the characteristically morphological changes of a dead cell *via* electron microscopy (Kerr et al., 1972). These specific morphological changes start with the initial shrinkage of the cell followed by detachment from the surrounding tissue. Thereafter, nuclear chromatin condenses and the DNA is cleaved. The plasma membrane bulges outward and loses its asymmetry as the cytosolic membrane component phosphatidylserine translocates to the outer surface of the cell (Kerr et al., 1972). Membrane blebs separate from the cell and form membrane-covered particles that contain cellular organelles and fragments of the nucleus, named apoptotic bodies. These are phagocytosed by neighboring cells, *via* phosphatidylserine serving as an "eat me" signal, without any local inflammatory reaction.

Apoptotic cell death is induced *via* different signaling pathways mediated by cysteine-dependent aspartate-specific proteases named caspases. By means of their role in apoptosis, caspases can be classified into two groups: initiator caspases, such as caspase-8 and -9, are activated in multicomponent protein complexes that consequently activate downstream effector caspases, such as caspase-3, afterwards, which subsequently triggers the apoptotic process invariable with its morphological changes described above.

Apoptosis can be initiated *via* an extrinsic/death-receptor-mediated or an intrinsic/mitochondria-associated pathway. The extrinsic pathway is initiated by binding of an external ligand to the corresponding death receptor, *i.e.*, FasR, tumor necrosis factor (TNF) receptor (R) or TNF-related apoptosis-inducing ligand receptor (TRAIL R), leading to conformational changes in the receptor and the recruitment of adaptor proteins. Within the multicomponent complex of receptor, ligand, and adaptor protein caspase-8 is activated.

The intrinsic pathway is initiated *via* the permeabilization of mitochondria, which harbors a multitude of apoptotic factors. Various stimuli such as oxidative stress, DNA damage or growth factor deprivation can initiate the cascade, which is characterized by a release of cytochrome C out of the mitochondria. Cytochrome C aggregates in a complex that activates caspase-9. Subsequently, both pathways lead to the activation of caspase-3 and are regulated by several pro- and anti-apoptotic factors. The inhibitor of apoptosis (IAP) family includes different members, such as XIAP, cIAP-1, cIAP-2, and survivin, which can bind directly and thereby inactivate caspases. Furthermore, cIAP-1 and -2 and XIAP serve as suppressors of Fas-mediated apoptosis through direct caspase-3 inhibition and modulation of the transcription factor NFκB (LaCasse et al., 1998). On the other hand, pro-apoptotic molecules such as second mitochondria-derived activator of caspases (SMAC) and high temperature requirement protein A2 (HtrA2), which are released from the mitochondria together with cytochrome C to bind IAPs and attenuate or even prevent their inhibitory effects on apoptosis (Van Loo et al., 2002). Different members of the B-cell lymphoma 2 (Bcl-2) family facilitate (Bad, Bax) or prevent (Bcl-2, Bcl-xL) the permeabilization of mitochondria and thereby function in a pro- or anti-apoptotic manner. The interaction of pro- and anti-apoptotic molecules is normally well balanced within the cell, but a shift to pro-apoptotic proteins leads to the induction of apoptosis and consequently cell death.

1.3. Syndecan-1

Sdc-1 is one of the four members of a family of transmembrane heparan sulfate proteoglycans that is typically present on the cell surface and carries chains of mainly heparan sulfate, but also chondroitin sulfate (Bernfield et al., 1999). Interestingly, Sdc-1 was also found to accumulate

in the nucleus and is assumed to regulate transcriptional activity directly (Brockstedt et al., 2002). Furthermore, intact Sdc ectodomains with their HS chains can be shed from the cell surface owing to proteolytical cleavage, leading to soluble effectors in the extracellular milieu and body fluid (Götte and Echtermeyer, 2003; Hayashida et al., 2006). Hence, the supposed biological character is rather complex and a main function at the cell surface implies the effect of Sdc-1 as a co-receptor and storage molecule for a wide range of ligands such as cytokines and growth factors affecting the stability, conformation or oligomerization state of ligands and receptors or increasing ligand concentration through their HS chains and regulating cell–cell interaction, cell migration, concentration, development, and tumorigenesis.

2. Apoptosis and syndecan-1 in the female reproductive tract throughout the menstrual cycle

2.1. Apoptosis

The role of apoptosis within the reproductive tract has already been described for multiple incidents regarding the reproductive physiology in human, most evidently, the distinct reduction in the number of ovarian follicles present at birth to adolescence and later in life the huge amount of follicles needed monthly to mature one follicle for ovulation. Both instances are associated with apoptotic follicle death at different developmental stages (Markström et al., 2002). The occurrence of endometrial cell apoptosis subject to the menstrual cycle is well described in the current literature. During the estrogen-stimulated proliferative phase of the cycle apoptosis is hardly present in the endometrium. The secretory phase is characterized by progesterone-induced glandular differentiation of the epithelium and stromal decidualization. Several studies described the presence of apoptotic endometrial glands and stromal cells from the beginning of the secretory phase throughout the receptive phase and further increasing in the late secretory phase with a maximum spread to almost all components of the functional layer during the menstrual phase (Kokawa et al., 1998a; Shikone et al., 1997; Vaskivuo et al., 2000). These observations led to the hypothesis that apoptosis might be responsible for endometrial breakdown during menstrual shedding, although other conflicting data describe the existence of only a few apoptotic stromal cells throughout the cycle (Vaskivuo et al., 2000; Von Rango et al., 1998) or alternatively an almost equal presence of apoptotic stromal cells at every phase of the cycle (Dmowski et al., 2001). Apoptosis-related receptors Fas and TNF are expressed in the endometrium with a constant level throughout the cycle (Tabibzadeh et al., 1995) and FasR being localized in the Golgi apparatuses during the proliferative phase and translocated to the cell membrane during the secretory phase (Yamashita et al., 1999). Nevertheless, the corresponding ligands FasL and TNF are expressed corresponding to cyclic, sex steroid-dependent changes in the endometrial stroma and epithelium, leading to an increase during the mid- and late secretory phase compared with the early proliferative phase (Selam, 2001; Tabibzadeh et al., 1995). Accordingly, the intrinsic anti-apoptotic protein Bcl-2 was shown to be highly expressed in the proliferative but not late secretory phase, indicating a role in protecting endometrial cells from apoptosis during the proliferative phase (Otsuki et al., 1994). Another hint of the involvement of the intrinsic and extrinsic apoptotic pathway in regulating menstrual tissue breakdown are studies describing an upregulation of active caspase-3, -8, and -9 and cytochrome C release from mitochondria in endometrial cells during the late secretory phase (Abe et al., 2006), accompanied by an increase in pro-apoptotic Bax (Tao et al., 1997). In early pregnancy the trophoblast expresses different factors, including cytokines, growth factors, FasL, and TRAIL which were assumed to be responsible for the apoptosis of activated decidual immune cells to maintain the immune privilege protecting the fetal allograft from the maternal immune system (Jerzak and Bischof, 2002). Several microarray analysis studies investigated the difference between undifferentiated stromal cells of the proliferative phase *vs.* differentiated, decidualized stromal cells of the mid-luteal phase showing a tremendous upregulation of genes involved in the immune response and negative regulation of growth. Furthermore, they emphasize the regulation of apoptosis-associated genes and receptors in the luteal phase endometrium implying the window of implantation. Herein, it was shown for example that TRAIL was upregulated in decidualized cells, which is known to function as a killer of activated lymphocytes and therewith possibly supports the proactive role of the decidua in protecting the invading embryo (Popovici et al., 2000).

Furthermore, the distinct role of the endometrium as a biosensor discriminating good *vs.* bad quality embryos was discussed recently. It was proposed that the endometrium might evolve strategies that prevent inappropriate investment in invasive but poor quality embryos and rather supports policies to avoid embryo implantation in such a case (Macklon and Brosens, 2014). Interestingly, it was observed that being in a co-culture with low-grade embryos endometrial stromal cells displayed selected inhibition of a high number of cytokines and growth factor secretion, whereas the co-culture with high-grade embryos had only a small impact on the decidualized stromal cells, suggesting the active role of the decidualized stromal cells in defeating embryo implantation. This specialized function seems to be strictly connected to the decidualization condition, as embryos triggered no response in undifferentiated stromal cells (Macklon and Brosens, 2014; Teklenburg et al., 2010). Furthermore, it was shown that stromal cells of women with recurrent miscarriage failed to discriminate between high- and low-quality embryos in contrast to stromal cells of healthy control individuals, which even arrested their baseline migration capacity in the presence of low-quality embryos (Weimar et al., 2012) fostering the idea of the human endometrium as a sentinel of embryo implantation.

2.2. Syndecan-1

It is prevalently described that Sdc-1 is expressed in the female reproductive tract (vaginal epithelium, cervix, and endometrium) and its expression seems to vary

throughout the menstrual cycle, implying the regulation of Sdc-1 *via* sex steroids. Nevertheless, the literature regarding the expression patterns of Sdc-1 demonstrates an inconsistency that might result from different staining methods and the application of various antibodies.

One study detected Sdc-1 in endometrial, luminal, and glandular epithelium, as well as in the stroma. Quantification of the expression revealed a significant decrease in Sdc-1 in the luminal epithelium during the proliferative phase, whereas stromal Sdc-1 expression significantly increased during the proliferative and ovulatory phase and no cycle-dependent changes in Sdc-1 were observed for the glandular epithelium (Lai et al., 2007). In another study an increase in Sdc-1 mRNA in whole endometrial tissue during the secretory phase was observed and further analysis *via* immunohistochemistry revealed the main localization of Sdc-1 on the apical surface of the glandular epithelium (Germeyer et al., 2007). Furthermore, one group noticed a rise in glandular and luminal epithelial Sdc-1 during the secretory phase, but failed to prove any statistical significance (Lorenzi et al., 2011). To summarize, hormonal regulation of Sdc-1 with an increase in the receptive phase epithelium is conceivable. On the contrary, the current literature provides an indication for a decrease in Sdc-1 in the stroma during the secretory phase. Accordingly, one group investigated Sdc-1 in placenta and decidua during early pregnancy and showed strong expression of Sdc-1 in the syncytiotrophoblast and placenta, but failed to detect Sdc-1 in the decidual stroma and glands (Jokimaa et al., 1998).

3. Implantation-associated apoptosis

To date, the literature on the role of endometrial apoptosis during embryo implantation has been contradictory: on the one hand the cell death of EECs after embryo contact has been intensely investigated, whereas on the other hand, studies on ESC apoptosis in humans are rare and most information has been derived from animal studies. Furthermore, even the literature on ESC apoptosis only summarizes conflicting data describing completely opposing trends, with either circumstantial apoptosis or resistance of human ESCs to apoptosis. In the following paragraphs, therefore, we try to shed some light on the debate.

3.1. Endometrial epithelium

After embryo attachment the endometrial epithelial layer is ruptured and becomes apoptotic within a short time period. A proposed mechanism for EEC destruction is *via* apoptosis mediated by the FasR/FasL system, as the trophoblast expresses FasL and the corresponding receptor was found on EECs and also on ESCs. *In vitro* confrontation culture of RL95-2 EECs and spheroids consisting of BeWo trophoblast cells revealed EEC apoptosis at the spheroid–EEC interface (Li et al., 2003). Furthermore, enhancement of EEC apoptosis and spheroid expansion was achieved by treatment with an antibody (ab) activating FasR and prevented with an ab neutralizing FasR (Hsu et al., 2008). An anti-Fas ab, which stimulated the death receptor equal to FasL, induced apoptosis *in vitro* in HHUA EECs and the pretreatment with transforming growth factor (TGF)-β1, interleukin (IL)-1β, and epidermal growth factor (EGF) enhanced Fas-mediated apoptosis in these cells (Tanaka and Umesaki, 2000). More recent studies revealed the role of the transmembrane protein trophinin in the elimination of EECs as a consequence of trophoblast adhesion. Both trophoblast cells and EECs express trophinin and hemophilic binding promotes invasive trophoblast differentiation and EEC apoptosis *via* protein kinase Cδ (Fritz et al., 2014).

Evidence from animal studies points out that apoptosis of EECs and ESCs plays a major role in the establishment of pregnancy in mice and rat (Gu et al., 1994; Tassell et al., 2000) in addition to rhesus monkey (Liu et al., 2005). One study in mice described in detail the regulation of apoptosis in the early stages of pregnancy in EECs compared with ESCs. They found the TNF-α receptor 1 (TNF R1) and the corresponding ligand in EECs, but not in ESCs, unlike proteins of the Bcl-2 family, which mediate the intrinsic apoptotic pathway, being expressed in ESCs but not in EECs. Decidual cells surrounding the embryo showed increasing pro-apoptotic Bad protein expression, whereas the adjacent stromal cells expressed anti-apoptotic Bcl-2 protein. Correspondingly, intrinsic caspase-9 was only activated in the decidua, but not in the epithelial endometrium. The authors concluded that two different signaling pathways mediate apoptosis in EECs and ESCs. First, the epithelial layer is eliminated fast *via* an extrinsic TNF R1-mediated pathway and further decidual apoptosis is regulated in a moderate spatial- and temporal-dependent fashion mediated by the intrinsic pathway (Joswig et al., 2003). These data are in accordance with the observation that EECs are rapidly and in a localized manner disrupted to completely, whereas in the stromal cell compartment the precise extent of invasion as a crucial factor for following proper pregnancy is limited and highly specific.

3.2. Endometrial stroma and decidua

In vivo studies on human ESC apoptosis during implantation are rare. ESCs were described to show an inveterate resistance toward Fas-mediated apoptosis *in vitro*, independent of their state of hormonal differentiation (nondifferentiated or decidualized [dESCs]), which was overcome by pretreatment of the cells with interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Fluhr et al., 2007). Furthermore, it was shown before that TGF-β1 induced apoptosis in primary cultured ESCs (Chatzaki et al., 2003), but did not affect the growth of endometrial epithelial cells originating from the cell line HHUA (Tanaka and Umesaki, 2000).

Human dESCs and trophoblast cells were also described to abundantly express heparin-binding (HB)-EGF during the early stages of pregnancy to promote trophoblast invasion and implantation (Leach et al., 1999). In accordance with these observations, HB-EGF was observed to mediate pro-survival signaling in ESCs (Fritz et al., 2014) and to stimulate dESC motility *in vitro*, suggesting that the cells might actively support implantation (Schwenke et al., 2013). Other studies showed the presence of scattered apoptotic dESCs in normal human first-trimester pregnancies *vs.* elevated apoptotic dESCs in cases of miscarriages

(Kokawa et al., 1998a). Additionally, a more limited extent of apoptotic dESCs as a consequence of ectopic pregnancy was demonstrated by the same group (Kokawa et al., 1998b). Furthermore, studies on endometriosis, a disease characterized by the presence of endometrial tissue located outside the uterus, described the presence of apoptotic ESCs in normal, healthy control individuals compared with a reduced number of apoptotic ESCs in individuals suffering from endometriosis. Additionally, the patients did not only show a decreased rate of apoptotic cells, but also a loss of the typical cyclic variability of apoptosis appearance (Dmowski et al., 2001; Roshangar et al., 2013).

We performed *in vitro* studies with the human immortalized human ESCs St-T1, which were purposefully treated with the cytokines IL-1β, IFN-γ, TNF-α, and TGF-β1, known to be secreted by the embryo during implantation, in addition to an anti-Fas ab replacing the FasL-bearing embryo, to clarify the role of apoptosis in human embryo implantation with emphasis on the endometrial stromal compartment. Interestingly, nondifferentiated ESCs were observed to be resistant toward apoptosis under all tested conditions and no induction of active caspase-3 was measurable. In contrast, decidualization with MPA and cAMP for 72 h led to a sensitization of dESCs and induction of apoptosis with the highest caspase-3 activation after treatment, with a combination of all cytokines and anti-Fas ab together (Boeddeker et al., data currently under review). The higher apoptotic susceptibility due to decidualization correlated with increased pro-apoptotic Bad, HTRA2, and SMAC and decreased anti-apoptotic cIAP-2 in dESCs compared with ESCs. Upstream of these proteins, the protein kinase Akt, which mediates cell survival *via* direct regulation of many different downstream effectors like IAPs or Bad, was found to be constitutively phosphorylated/active in nondifferentiated ESCs, whereas decidualization led to a decrease in pAkt. Increased pAkt in nondifferentiated ESCs was suggested to result from an autocrine activation *via* secreted HGF (Sugawara et al., 1997) and corresponding activation of the HGF/Met signaling pathway, leading to enhanced activation of Akt. The influence of decidualization upon Akt activation has been described before, albeit indirectly by observation of a PTEN protein increase, which functions as an inhibitor of the Akt pathway, in dESCs during the late secretory phase (Mutter et al., 2000). Additionally, Forkhead box protein O1 (FoxO1a) is a transcription factor in the cell nucleus that participates in the induction of apoptosis (Calnan and Brunet, 2008). Phosphorylation of FoxO1a *via* pAkt leads to exclusion of FoxO from the nucleus to the cytoplasm, which is associated with reduced transcriptional activity. It has been reported that un-phosphorylated FoxO1a accumulates in the nuclei of dESCs *in vivo* and this correlates with suppression of the Akt pathway upon decidualization (Gellersen and Brosens, 2003). Thus, decreased pAkt in dESCs compared with undifferentiated endometrial stromal cells, as seen in our study, may be a consequence of successful decidualization. In summary, decidualization that is necessary for proper embryo implantation provides the opportunity for the ESCs to induce apoptosis in reaction to embryonic stimuli mediated *in vitro via* decreased activation of Akt, which also suggests that stromal cell apoptosis might play an important role implantation *in vivo*.

3.3. Endometrial vascular endothelial and smooth muscle cells

Endometrial spiral arteries evolve in the secretory phase of the menstrual cycle and are progressively transformed during embryo implantation, including a strong dilatation of the vessel and the loss of smooth muscle and endothelial cells to ensure the nutrition of the embryo during this early phase of the pregnancy. Different studies hypothesize the role of vascular cell apoptosis to facilitate spiral artery remodeling. *In vitro* experiments revealed apoptosis of endothelial and vascular smooth muscle cells *via* trophoblast-derived secretion products, activating the FasR and TRAIL R system (Harris et al., 2006; Keogh et al., 2007). Immunohistochemical examination of first-trimester placental bed samples indicated the presence (Smith et al., 2009) and absence (Bulmer et al., 2012) of apoptotic vascular smooth muscle cells as a consequence of spiral artery remodeling. Whereas the latter study postulated that the cells migrate away from the vessel, other studies correlated the failure in detecting apoptosis with the rapidity of cells dying and being removed *via* a large number of phagocytes within the decidua (Smith et al., 2009; Whitley and Cartwright, 2009).

4. The role of syndecan-1 in apoptosis

The influence of Sdc-1 on apoptosis has been described previously for various cancer cells and revealed a Janus-faced attitude, as it exhibited opposed effects in a cell-type-dependent manner. For cancer types of the female reproductive tract it has been reported, that Sdc-1 was decreased in cervical cancer and an inverse correlation between Sdc-1 expression and metastasis was shown (Numa et al., 2002), whereas the contrary was observed for breast (Barbareschi et al., 2003) and ovarian (Davies et al., 2004) cancer, where overexpressed Sdc-1 correlated with high proliferation, invasion, an aggressive phenotype, and a poor clinical prognosis. Studies with endometrial cancer cells showed that Sdc-1 expression was increased compared with normal endometrium. Further overexpression *via* transfection of cells with Sdc-1 cDNA promoted the proliferation of the cells *via* activation of nuclear factor NFκB (Oh et al., 2009), whereas the silencing of Sdc-1-induced apoptosis correlated with decreased activation of pro-survival Akt (Choi et al., 2007).

The possible role of Sdc-1 in influencing implantation-associated apoptosis was revealed by *in vitro* studies in our laboratory, indicating that Sdc-1 modulates the fine tuning of apoptosis in EECs and ESCs. First, we revealed that Sdc-1 knockdown (kd) increased the apoptotic sensitivity toward embryonic stimuli (IL-1β, IFN-γ, TNF-α, TGF-β1, and anti-Fas ab) in the EEC line RL95-2 in connection with a downregulation of several anti-apoptotic proteins, *e.g.*, cIAP-1/-2, XIAP, and survivin, and upregulation of the death receptors FasR and TRAIL R 2 in EECs with Sdc-1 kd compared with normal EECs (Boeddeker et al., 2014) (Table 1). Second, our studies with ESCs revealed the resistance of nondifferentiated cells toward apoptosis, which was negotiated after decidualization, as already described above. Interestingly, nondifferentiated ESCs with

Table 1
Changes in the expression of apoptosis-related proteins in endometrial epithelial cells (EECs) with syndecan-1 knockdown (Sdc-1 kd; first column) and alterations in endothelial stromal cells (ESCs) upon decidualization (second column), changes in ESCs due to Sdc-1 kd (third column) and changes due to a combination of Sdc-1 kd and decidualization (fourth column). Upregulated proteins are indicated using an upward aligned arrow (↑); downregulated proteins are indicated with a downward arrow (↓).

		EECs	ESCs		
		Sdc-1 kd	Decidualization	Sdc-1 kd	Sdc-1 kd and decidualization
Pro-apoptotic	Bad		↑	↑	↑
	Bax				
	HTRA2		↑		↑
	SMAC		↑		
Death receptors	TRAIL R1				
	TRAIL R2	↑	↑		↑
	FasR	↑	↑		↑
	TNF R1	↓			
	Bcl-2				
Anti-apoptotic	cIAP-1	↓	↓	↓	
	cIAP-2	↓			
	XIAP	↓		↓	↓
	Livin		↓		↓
	Survivin	↓			
	Clusterin	↓			
	HO-1				
	HO-2	↓			
	HSP27	↓	↑		↑
	HSP70	↓			

Sdc-1 kd already induced apoptosis after treatment with embryonic stimuli independently of decidualization, but the effect was additive after decidualization of ESCs with Sdc-1 kd, which showed the highest activation of caspase-3. The higher apoptotic susceptibility due to Sdc-1 kd in ESCs also correlated with increased Bad and decreased cIAP-1 and XIAP, and correspondingly decreased activation of pro-survival Akt in ESCs with Sdc-1 kd, compared with wild-type ESCs (Table 1). The influence of Sdc-1 on Akt signaling has already been described for endometrial cancer cells and prostate cancer cells, where Sdc-1 kd decreased anti-apoptotic pAkt and induced apoptosis (Choi et al., 2007). Furthermore, it is known that Sdc-1 orchestrates HGF/Met signaling, resulting in enhanced activation of signaling pathways involved in the control of cell proliferation and survival, such RAS/MAPK and PI3K/Akt (Derksen et al., 2002). Therefore, constitutive activation of Akt in normal ESCs that was already hypothesized as an autocrine mechanism (Yoshida et al., 2004) mediating apoptotic resistance, may be interrupted in ESCs lacking Sdc-1.

Interestingly, XIAP was only reduced in Sdc-1 kd cells and not dESCs compared with nondifferentiated ESCs. This anti-apoptotic protein is known to mediate first-trimester trophoblast resistance to Fas-mediated apoptosis regardless of expressing both FasR and FasL (Straszewski-Chavez et al., 2004), which is interesting and should be kept in mind, as dESCs also express both FasR and FasL (Harada et al., 2004) and the higher apoptotic susceptibility due to Sdc-1 kd seems to be connected to decreased XIAP.

In all endometrial cells EECs, ESCs, and dESCs, FasR was increased after treatment with the embryonic stimuli IL-1β, IFN-γ, TNF-α, TGF-β1, and anti-Fas ab (Table 1), which may sensitize the cells to Fas-mediated apoptosis and showed the potentially important role for embryo–maternal FasL/FasR interaction during implantation. Furthermore, another death-receptor, TRAIL R1/2, was significantly upregulated in cytokine-and anti-Fas ab-treated EECs, whereas TRAIL R2 increased in treated and decidualized ESCs with and without Sdc-1 kd (Boeddeker et al., data currently under review). This indicates an additional role for the TRAIL/TRAIL R system besides FasL/FasR in implantation-correlated apoptosis. This hypothesis is supported by the fact that the ligand TRAIL is upregulated upon *in vitro* decidualization (Popovici et al., 2000) and expressed in dESCs in first-trimester placental biopsies combined with the corresponding receptors TRAIL R1/2 (Phillips et al., 1999). Fig. 1 depicts the hypothetical interactions of Sdc-1 in EECs and ESCs as discussed herein.

5. The role of apoptosis and syndecan-1 in implantation failure and pregnancy disorders

Several clinical studies revealed that certain pregnancy complications and disorders are associated with modified placental development due to apoptosis. Particularly in cases of miscarriage (Kokawa et al., 1998a), unexplained recurrent spontaneous abortion (Wei et al., 2014), preeclampsia (Allaire et al., 2000; Cali et al., 2013; Prusac et al., 2011), HELLP syndrome (Cali et al., 2013; John et al., 2013; Prusac et al., 2011), IUGR (Börzsönyi et al., 2013; Cali et al., 2013; Heazell et al., 2011; Roje et al., 2011), and in endometrium with out-of-phase receptive alignment, which relates to repeated implantation failure (Meresman et al., 2010) an increased number of apoptotic endometrial and placental cells was shown, whereas a diminished number of apoptotic cells was observed in cases of ectopic pregnancy outside the uterus (Kokawa et al., 1998b).

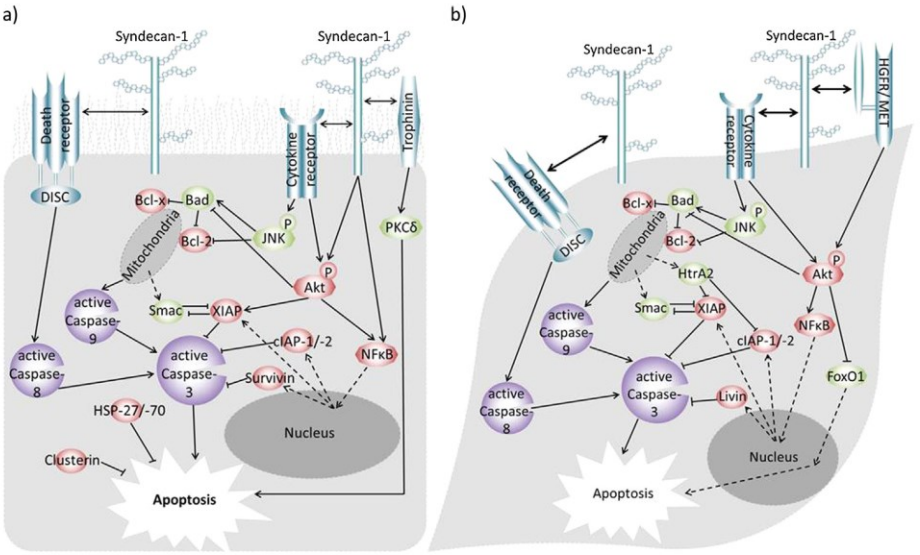

Fig. 1. The hypothetical signaling cascade among Sdc-1, death, and cytokine receptors, in addition to trophinin, HGFR, and pro- and anti-apoptotic proteins, is depicted in (a) EECs and (b) ESCs. The hypothetical interaction between Sdc-1 and receptors is depicted by a double arrow ↔, activating or promoting processes are indicated using a single arrow →, translocations are indicated using a discontinuous arrow, blocking or inhibiting processes are indexed with a stop arrow —I, pro-apoptotic proteins are labeled green, and anti-apoptotic proteins are labeled red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Clinical studies to clarify the role of Sdc-1 in the development of pregnancy disorders that result from inadequate implantation again revealed a Janus-faced attitude of Sdc-1 and were therefore extremely divergent in their findings. On the one hand, diminished Sdc-1 expression correlated with idiopathic fetal growth restriction (Chui et al., 2012), preeclampsia (Heyer-Chauhan et al., 2013), and HELLP syndrome (Norwitz, 2007), and according to their findings elevated Sdc-1 was connected with a reduced risk of preterm labor (Schmedt et al., 2012). On the other hand, increased syncytiotrophoblastic Sdc-1 expression was associated with preeclampsia and HELLP syndrome as well (Szabo et al., 2013). A fundamental limitation of these studies was the restricted disposability of samples of patients and healthy controls at early pregnancy.

The data currently available in the literature and our previous observations indicate that apoptosis plays a major role in the female reproductive tract with regard to all different requirements throughout the menstrual cycle, including decidualization, implantation or menstrual shedding of the nonpregnant endometrium.

Furthermore, our studies reveal that Sdc-1 seems to attenuate the apoptotic signal triggered by embryonic stimuli *in vitro* in ESCs and EECs, indicating that Sdc-1 may act as a modulator of invasion depth, thereby representing a crucial factor for successful pregnancy. Sdc-1 seems to exert its influence on implantation *via* regulating ESC and especially EEC apoptosis, even if the exact Sdc-1 influence on apoptosis induction has not been fully understood so far. To summarize, apoptosis as an early regulator of successful implantation seems to be multifactorially regulated and represents a possible starting point for investigating and discovering strategies for patients with recurrent implantation failure in assisted reproductive therapies and patients with pregnancy diseases associated with inadequate implantation.

Conflict of interest

None.

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4. Findings and wider implications

In the present work the role of maternal endometrial apoptosis triggered by embryonic and implantation-related stimuli has been elucidated by performing *in vitro* studies with EEC and ESC lines treated with cytokines known to be secreted during implantation as well as an anti-Fas ab replacing the FasL-bearing embryo mimicking the embryo-maternal contact. The findings suggest an induction of apoptosis in EECs and decidualized ESCs due to the embryo contact during implantation, supposedly to eliminate EECs to facilitate the embryonic invasion via a temporary and intense apoptotic induction and finally to regulate the depth of implantation in ESCs by a more temporal and local restricted apoptotic reaction compared to EECs. Interestingly, non-decidualized ESCs showed a resistance toward apoptosis, which was reversed by decidualization. Decidualization is a known important process to develop a receptive endometrium that prepares the window of implantation which is the only time in the menstrual cycle where a pregnancy can be successfully established in human.

Equally important, the role of Sdc-1 in attenuating the apoptotic signal in EECs and ESCs has been discovered, generating a new EEC line with a stable kd for Sdc-1 and using a Sdc-1 kd ESC line, which was generated by our laboratory before. These findings indicate that Sdc-1 may be involved in the fine-tuning of endometrial cell apoptosis during embryo implantation. It is probably influencing the precisely adjusted process of implantation and thus the pregnancy outcome, because excessive apoptosis as observed in the Sdc-1 kd EECs and ESCs could provoke an incorrect implantation with complications and disorders leading to pregnancy complications or even maternal and fetal death.

Limitations of the study are the use of cell lines and the mixture of cytokines and anti-Fas ab that served as a model for the embryo-maternal contact. Thus, further studies are scheduled reflecting the *in vivo* situation more closely. More detailed, the cultivation and investigation of primary cells directly derived from endometrial tissue will be targeted, to exclude a possible influence of the immortalization of the cell lines on the apoptotic inducibility. A transient kd of Sdc-1 via siRNA in the primary cells will help to gain new insights into the influence on apoptosis within these cells. Current preliminary studies regarding the isolation, cultivation and the transient kd of Sdc-1 are displayed in Figure 4.1.

Furthermore, a novel array detecting 102 cytokines (R&D systems, Minneapolis, USA) will be applied with cell supernatants from human blastocysts being *in vitro* cultured for 5 days in our IVF-laboratory to modify and improve the model to mimic the embryo contact.

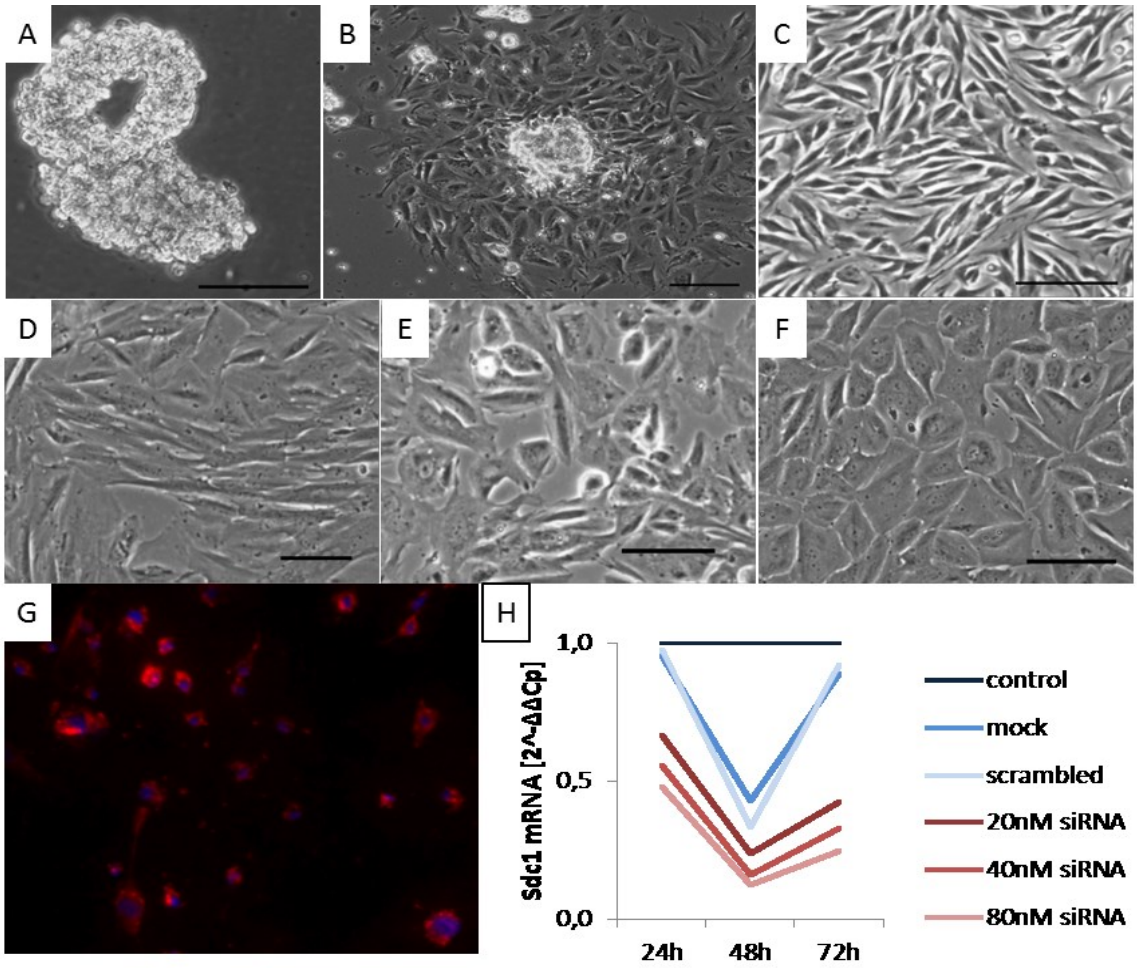

Fig. 4.1: Preliminary studies with primary endometrial cells. (A) Epithelial, glandular tube at day 1 after isolation, (B) after 5 days epithelial cells grow out from the tubular organization. (C) Endometrial stromal cells at day 1 after isolation. (D-E) *In vitro* decidualization of primary stromal cells treated with progesterone for 3 days (E) and 6 days (F) versus 6 days untreated (D). (G/H) Transient transfection of primary stromal cells. (G) Expression of Cy5-labeled scrambled siRNA 48h after transfection and (H) fold induction of Sdc-1 mRNA after transfection with siRNA.

Since research on embryonic implantation is limited to animal models or *in vitro* experiments with human cells, novel and alternative 3D- and co-culture systems mimicking the physiological architecture of the endometrium are targeted to approximate the *in vivo* situation. In preliminary studies, different cell culture systems were established and further experiments will utilize the potential of these methods. In detail, with the help of a coated, low cell binding 96-well plate with round bottom a defined number of trophoblastic cells was conglomerated to dense and globular spheroids, which were co-cultured with endometrial cell monolayers. The spheroid outgrowth was monitored and apoptotic cells at

the spheroid/endometrial cell interface were stained with specific fluorescent dyes. Moreover, a 3D-co-culture system of a thick layer of ESC, a thin EEC layer and a trophoblastic cell spheroid on top was established. For this purpose ESCs were incorporated into a scaffold of fibrin (from human plasma) and agarose and solidified in a special cell insert with a membrane bottom side to guarantee the all-around maintenance of the cells with medium, followed by the application of EECs and trophoblastic spheroids. For histological characterization the culture system was removed from the insert, fixed, embedded, and sectioned like a native tissue (Figure 4.2). This system provides novel opportunities regarding the investigation of factors influencing trophoblast invasion, enclosed Sdc-1, signaling pathways or even environmental conditions and drugs.

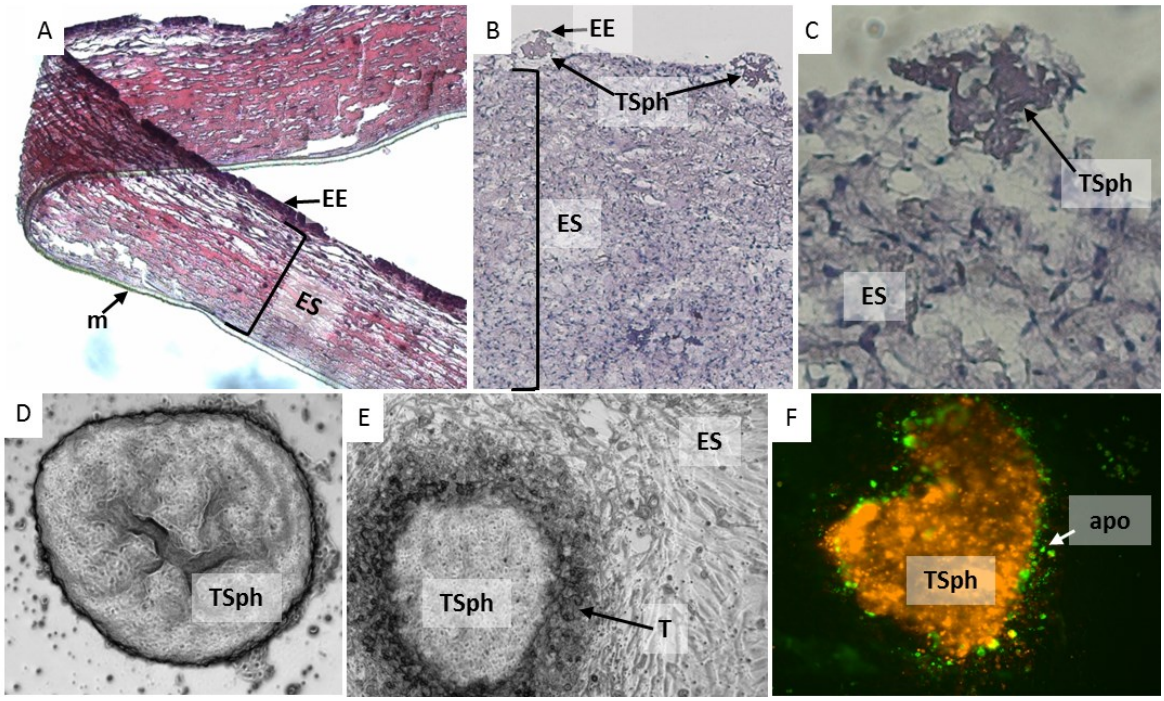

Fig. 4.2: Preliminary studies with 3D- and co-cultures. (A) Paraffin-embedded section of a 3D-culture with endometrial epithelial [EE] and stromal cells [ES], the membrane of the insert [m] is visible at the bottom. (B) Paraffin-embedded section of a 3D-culture with trophoblast spheroids [TSph]. (C) Detailed view of TSph. (D) TSph after 24h on a tissue culture dish vs. (E) on ES monolayer. The outgrowth of trophoblastic cells [T] is visible. (F) TSph stained with cell tracker orange on an unstained ES monolayer. Cells at the TSph/ES interface are stained with Annexin-FITC (green) to detect apoptotic cells [apo].

In the context of the present work, new targets supposedly mediating endometrial cell interaction with the embryo were deciphered, including the TRAIL- /TRAIL R system as well as FoxO1a and further studies will give a deeper insight in the regulation of a proper implantation.

In vivo investigations of human implantation sides are not feasible for ethical reasons, so *in vivo* studies are planned with wildtype *vs.* Sdc-1 ko mice. From murine uteri the

implantation chambers and fetuses are supposed to be extracted at day 4 to 15 *post coitum* and investigated via immunohistochemistry and -fluorescence with regard to apoptotic markers such as DNA strand breaks and related proteins including FasR, TRAIL, IAPs and Bad. These experiments will help to decipher a possible key role of Sdc-1 on the regulation of implantation depth via influencing endometrial apoptosis in mice.

Taken together, the present work features answers regarding endometrial cell apoptosis through embryonic signals and Sdc-1 regulating the apoptotic signal, but also paves the way for further experiments to gain deeper insights in the complex regulation of embryo implantation in human.

5. Summary

Embryo implantation (invasion and nidation) into the inner wall of the female uterus constituted by the endometrium is the perquisite for following successful pregnancy. It requires a good quality embryo, a receptive endometrium of highly differentiated epithelial and stromal cells and a synchronized molecular dialog between embryo and maternal endometrium. We hypothesized that programmed cell death (apoptosis) of maternal endometrial epithelial (EEC) and stromal (ESC) cells as a consequence of the embryo contact plays a crucial role in pioneering the embryos entrance into the maternal system and in regulation of the implantation depth.

Syndecan-1 (Sdc-1) is a cell surface molecule expressed on endometrial cells and highly upregulated in the window of implantation, characterizing the time period of the menstrual cycle with the endometrium only being receptive for the embryo implantation. Sdc-1 functions as a co-receptor for cytokines but also directly influences signaling pathways, such as regulators of apoptosis. An altered Sdc-1 expression was already correlated with pregnancy disorders, which in turn have been associated with an insufficient implantation. Therefore, an involvement of Sdc-1 in the implantation process via regulation of EEC and ESC apoptosis is most likely.

EEC and ESC lines were cultivated *in vitro* and pendants with a stable Sdc-1 knock downs (kd) were generated. ESCs were further decidualized with hormones to achieve the *in vivo* situation of a receptive endometrium. Treatment with the four cytokines interleukin (IL)-1β, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1 as well as an anti-Fas antibody (IITT+F) were applied as a suitable model to mimic embryo contact. Non-decidualized ESCs revealed a resistance toward apoptosis induced by IITT+F, which was reversed upon decidualization, whereas ESCs with Sdc-1 kd were sensitive to the apoptotic signal independent of decidualization. Sdc-1 kd led to a higher apoptotic inducibility in ESCs and EECs, which was correlated with an altered expression of pro- and anti-apoptotic proteins, in particular the inhibitor of apoptosis family and pro-apoptotic Bad, as well as death receptors FasR and TRAIL R. The apoptotic resistance of non-decidualized ESCs was associated with a sustained activation of the pro-survival signaling molecule Akt and according to this decidualization and Sdc-1 kd, which facilitates the induction of apoptosis, deactivates Akt.

Taken together, EECs and decidualized ESCs induce apoptosis after *in vitro* treatment with embryonic stimuli, indicating a potential role for maternal apoptosis during implantation. Furthermore, Sdc-1 seems to attenuate the apoptotic signal, suggesting that Sdc-1 may act as a crucial factor for successful pregnancy affecting implantation via regulating endometrial apoptosis. Especially with regards to the development of novel therapeutic strategies for obstetrics and reproductive medicine this is of immense importance.

6. Zusammenfassung

Die embryonale Implantation (Einwandern und Einnistung) in die innere Wand des weiblichen Uterus (genannt Endometrium) als eine Grundvoraussetzung für eine darauf folgende erfolgreiche Schwangerschaft benötigt einen gut entwickelten Embryo, ein rezeptives (aufnahmebereites) Endometrium mit differenzierten epithelialen und stromalen Zellen und einen hoch abgestimmten molekularen Dialog zwischen Embryo und mütterlichen Endometrium. Wir stellten die Hypothese auf, dass der programmierte Zelltod in Form von Apoptose mütterlicher, endometrialer Epithel- (EEZ) und Stromazellen (ESZ), hervorgerufen durch den Embryokontakt, eine entscheidende Rolle dabei spielt, dem Embryo den Weg in das mütterliche System zu ebnen, sowie die Implantationstiefe zu regulieren.

Syndecan-1 (Sdc-1) ist ein Zelloberflächenmolekül, dass auf endometrialen Zellen exprimiert wird und während des Implantationsfensters, dem Zeitpunkt innerhalb des Menstruationszyklus, in dem das Endometrium aufnahmebereit für den Embryo ist, stark hochreguliert wird. Sdc-1 wirkt als Ko-Rezeptor für Zytokine, kann aber auch Apoptose-regulierende Signalwege direkt beeinflussen. Eine veränderte Sdc-1 Expression wurde bereits mit Schwangerschaftserkrankungen in Verbindung gebracht, welche wiederum mit einer unzureichenden Implantation assoziiert werden. Aus diesem Grund ist ein Einfluss von Sdc-1 auf die Implantation durch Regulierung von EEZ und ESZ Apoptose höchstwahrscheinlich.

EEZ und ESZ Linien wurden *in vitro* kultiviert und Gegenstücke mit einem stabilen Sdc-1 knock down (kd) generiert. ESZ wurden weiterhin durch Hormone dezidualisert, um den *in vivo* Zustand eines rezeptiven Endometriums nachzuahmen. Es wurde eine Behandlung mit den vier Zytokinen Interleukin (IL)-1β, Interferon (IFN)-γ, Tumornekrosefaktor (TNF)-α,

transforming growth factor (TGF)-β1, sowie einem anti-Fas Antikörper (IITT+F) als adäquates Modell, um einen Embryokontakt nachzubilden, angewandt. Undezidualiserte ESZ offenbarten eine Resistenz gegenüber Apoptose durch IITT+F, welche durch Dezidualisierung aufgehoben wurde, während ESZ mit Sdc-1 kd unabhängig einer Dezidualisierung sensitiv gegenüber dem apoptotischen Signal waren. Der Sdc-1 kd führte zu einer höheren Auslösbarkeit der Apoptose in EEZ und ESZ und dies wurde mit einer veränderten Expression pro- und anti-apoptotischer Proteine, z.B. der inhibitor of apoptosis Familie, dem pro-apoptotischen Bad und den Todesrezeptoren FasR und TRAIL R, in Verbindung gebracht. Die Apoptoseresistenz der undezidualisierten ESZ wurde mit einer konstitutiven Aktivität des *pro-survival* Signalmoleküls Akt in Verbindung gebracht und dementsprechend führte eine Dezidualisierung und der Sdc-1 kd, wodurch Apoptose ermöglicht wurde, zu einer De-aktivierung von Akt.

Zusammenfassend leiten EEZ und ESZ die Apoptose nach *in vitro* Behandlung mit embryonalen Stimuli ein, was auf eine mögliche Rolle mütterlicher Apoptose während der Implantation hinweist. Weiterhin scheint Sdc-1 das apoptotische Signal abzumildern, was darauf schließen lässt, dass Sdc-1 ein äußerst wichtiger Faktor für eine erfolgreiche Schwangerschaft ist, der die Implantation durch Regulierung endometrialer Apoptose beeinflusst. Dies ist gerade auch in Hinsicht auf die Entwicklung neuer therapeutischer Strategien für die Geburtshilfe und Reproduktionsmedizin von immenser Bedeutung.

Appendix

2. Scientific Curriculum Vitae

Professional experience

6/2010 – today	PhD student - department of OB/ GYN and REI (UniKiD) at the Heinrich-Heine University Medical Center, Duesseldorf, Germany
11/2009 – 3/2010	Research Assistant – department of Toxicology at the Heinrich-Heine University, Duesseldorf, Germany

Education

12/2004 – 9/2009	Studies of biology, Heinrich-Heine University, Duesseldorf, Germany Degree: Diploma with distinction

Publications in peer reviewed journals

- Decidualization and syndecan-1 knock down sensitize endometrial stromal cells to apoptosis induced by embryonic stimuli.
  Boeddeker SJ, Baston-Buest DM, Fehm T, Kruessel J, Hess A. PLoS One. 2015 Apr 1;10(4):e0121103. doi: 10.1371/journal.pone.0121103. eCollection 2015.

- The role of apoptosis in human embryo implantation.
  Boeddeker SJ, Hess AP. J Reprod Immunol. 2015 Feb 28. pii: S0165-0378(15)00032-7. doi: 10.1016/j.jri.2015.02.002.

- Syndecan-1 knockdown in endometrial epithelial cells alters their apoptotic protein profile and enhances the inducibility of apoptosis.
  Boeddeker SJ, Baston-Buest DM, Altergot-Ahmad O, Kruessel JS, Hess AP. Mol Hum Reprod. 2014 Jun;20(6):567-78. doi: 10.1093/molehr/gau009. Epub 2014 Jan 29.

- CXCL1 Expression in Human Decidua in vitro is Mediated via the MAPK Signalling Cascade.
  Baston-Büst DM, Schanz A, Boeddeker SJ, Altergot-Ahmad O, Krüssel JS, Rein D, Hess AP, Cytokine. 2013 Oct;64(1):79-85. Epub 2013 Aug 15.

- Effects of a Syndecan-1 knock down on cellular total protein composition in human decidualized endometrial stromal cells.
  Baston-Buest DM, Fehr D, Boeddeker SJ, Fehm T, Kruessel JS, Hess AP. J Reprod Dev (under review)

- The influence of matrix metallopoteinases MMP7 and ADAMTS6 on the etiology of tubal implantation in human.
  Beyer I, Martignoni F, Boeddeker SJ, Baston-Buest DM, Kruessel JS, Fehm TN, Hess AP. Fertil Steril (under review)

- The impact of Syndecan-1 on MAPK and Wnt signaling in human endometrial stromal cells.

Altergot-Ahmad O, Baston-Buest DM, Boeddeker SJ, Fehm T, Kruessel JS, Hess AP. Mol Hum Reprod (under review)

Abstracts (a), oral presentations (o) and posters (p)

- Die Rolle von Syndecan-1 bei der Apoptose endometrialer Epithel- und Stromazellen (a,o)
  Boeddeker SJ, Baston-Büst DM, Krüssel JS, Fehm TN, Hess AP
  14th Annual meeting of the Society Molecular Biology of the German Society of Gynecologic Endocrinology and Reproductive Medicine (DGGEF) and the German Society for Reproductive Medicine (DGRM), 11/2014, Muenster, Germany

- Molecular characterization of the Syndecan-1 knock-out mouse regarding its reproductive phenotype and offspring (a,o)
  Gougoula C, Baston-Buest DM, Boeddeker SJ, Krüssel JS, Fehm TN, Hess AP
  14th Annual meeting of the Society Molecular Biology of the German Society of Gynecologic Endocrinology and Reproductive Medicine (DGGEF) and the German Society for Reproductive Medicine (DGRM), 11/2014, Muenster, Germany

- Apoptosis of endometrial epithelial cells by embryonic stimuli and influence of Syndecan-1 (a,p)
  Boeddeker SJ, Baston-Buest DM, Altergot-Ahmad O, Kruessel JS, Hess AP
  61st Annual Scientific Meeting of the Society for Gynecological Investigation (SGI), 3/2014, Florence, Italy

- Human endometrial stromal cells express CXCL1 *in vitro* through different signaling pathways (a,p)
  Altergot-Ahmad O, Boeddeker SJ, Baston-Buest DM, Kruessel JS, Hess AP
  30th Annual Meeting of the European Society of Human Reproduction and Embryology (ESHRE), 7/2014, Munich, Germany

- Apoptose endometrialer Epithelzellen durch embryonale Stimuli und Einfluss von Syndecan-1 (a,p)
  Böddeker SJ, Altergot-Ahmad O, Baston-Büst DM, Krüssel JS, Hess AP
  DVR congress (Umbrella Organization Reproductive Medicine), 12/2013, Muenster, Germany

- Die CXCL1-Expression wird in endometrialen Stromazellen unabhängig von Syndecan-1 über den MAPK-Signaltransduktionsweg reguliert (a,p)
  Altergot-Ahmad O, Boeddeker SJ, Baston-Buest DM, Kruessel JS, Hess AP
  DVR congress (Umbrella Organization Reproductive Medicine), 12/2013, Muenster, Germany

- Gibt es einen Tod vor dem Leben? Apoptose endometrialer Stroma- und Epithelzellen durch embryonale Stimuli (a,o)
  Boeddeker SJ, Baston-Büst DM, Altergot-Ahmad O, Krüssel JS, Hess AP
  13th Annual meeting of the Society Molecular Biology of the German Society of Gynaecologic Endocrinology and Reproductive Medicine (DGGEF) and the German Society for Reproductive Medicine (DGRM), 11/2013, Duesseldorf, Germany

- Knock down of Syndecan-1 reduces the expression of anti-apoptotic proteins in the endometrial epithelial cell line RL95-2 (a)
  Boeddeker SJ, Baston-Buest DM, Altergot-Ahmad O, Hirchenhain J, Kruessel JS, Hess AP
  29th Annual Meeting of the European Society of Human Reproduction and Embryology (ESHRE), 6/2013, London, UK

- Syndecan-1 does not regulate the expression of CXCL1 through the MAPKinase signaling pathway in decidualized endometrial stromal cells (a,p)
  Altergot-Ahmad O, Baston-Buest DM, Boeddeker SJ, Kruessel JS, Hess AP
  The Endocrine Society's 95th Annual Meeting, 6/2013 San Fransisco, USA

- Der Embryosurrogatmarker IL-1β stimuliert die Expression des chemokine ligand 1 (CXCL1) in dezidualisierten endometrialen Stromazellen (a,p)
  Baston-Buest DM, Boeddeker SJ, Altergot-Ahmad O, Fehr D, Schanz A, Janni W, Krüssel JS
  59th Congress of the German Society for Gynecology and Obstetrics (DGGG), 10/2012, Munich, Germany

- Angiogenic factor composition of decidualized endometrial stromal cells is modified by knock down of Syndecan-1 by imitation of embryo contact (a,p)
  Baston-Buest DM, Boeddeker SJ, Altergot-Ahmad O, Ziegler D, Janni W, Krüssel JS
  Joint International Congress American and European Society for Reproductive Immunology, 5/2012, Hamburg, Germany

- Evaluation des Chemokinrezeptor CXCR2 knock downs in endometrialen Stromazellen mittels Quantigene® Plex (a,o)
  Altergot-Ahmad O, Baston-Buest DM, Boeddeker SJ, Ziegler D, Kruessel JS, Janni W, Hess AP
  11th Annual meeting of the Society Molecular Biology of the German Society of Gynaecologic Endocrinology and Reproductive Medicine (DGGEF) and the German Society for Reproductive Medicine (DGRM), 12/2011, Duesseldorf, Germany

- Einfluss des Ko-Rezeptors Syndecan-1 auf die Expression Apoptose-beteiligter Proteine in humanen dezidualisierten endometrialen Stromazellen (a,o)
  Boeddeker SJ, Baston-Büst DM, Altergot O, Krüssel JS, Janni W, Hess AP
  10th Annual meeting of the Society Molecular Biology of the German Society of Gynaecologic Endocrinology and Reproductive Medicine (DGGEF) and the German Society for Reproductive Medicine (DGRM), 11/2010, Duesseldorf, Germany

- Toxicological effects of the anthraquinone derivative Altersolanol A isolated from *Stemphylium globuliferum* (a,p)
  Boeddeker SJ, Debbab A, Rohrig R, Proksch P, Waetjen W
  51st Meeting of the german society for experimental and clinical pharmacology and toxicology (DGPT) 3/2010, Mayence, Germany

Awards and Honors

- Nominated for the 16th Royan International Research Award, 2nd September 2015 in Tehran, Iran

- Es war einmal… die Geschichte der Syndecan-1 ko Mause – ein Wissenschaftsmärchen der Geschwister UniKiD (a,o)
  Baston-Büst DM, Boeddeker SJ, Hirchenhain J, Hess AP
  28th Annual meeting of the German IVF-Centres, 12/2014 Stuttgart, Germany
  Basic Science Award in category science slam

- Embryonale Sekretionsprodukte und Oberflächenproteine initiieren Apoptose in endometrialen Epithel- und Stromazellen und regulieren damit die Implantationstiefe (a, o)
  Boeddeker SJ, Baston-Büst DM, Krüssel JS, Fehm TN, Hess AP

60th Congress of the German Society for Gynecology and Obstetrics (DGGG), 10/2014, Munich, Germany
Awarded with the Basic Science Prize

- Bestimmung von Syndecan-1 Expression bei IVF/IVF-ICSI-Patientinnen in Korrelation mit Schwangerschafts- und Baby-take-home-Rate zur Etablierung eines günstigen, vor Ort durchführbaren Tests zur Bestimmung der endometrialen Rezeptivität und Steigerung der individuellen Schwangerschaftsrate
  Project proposal for the Idea Award 2015 (best draft for a clinical-scientific project in the Department of OB/ GYN and REI) in the University Clinics of Duesseldorf, Germany

- Syndecan-1 knock-down alters apoptotic suseptibility to embryonic stimuli in the human endometrial epithelial cell line RL95-2 (a, p, o)
  Boeddeker SJ, Baston-Buest DM, Altergot-Ahmad O, Kruessel JS, Hess AP
  30th Annual Meeting of the European Society of Human Reproduction and Embryology (ESHRE), 7/2014, Munich, Germany
  Selected for the Basic Science Award for poster presentation

- Implantation und die Moleküle des Schicksals (a, o)
  Boeddeker SJ, Baston-Büst DM, Krüssel JS, Hess AP
  26th Annual meeting of the German IVF-Centres, 12/2012 Cologne, Germany
  Basic Science Award in category science slam

Membership

European Society of Human Reproduction and Embryology (ESHRE) since 2012

Further trainings

3/2015	„Wissenschaftliche Bildbearbeitung und Analyse“, medRSD (Medical Research School Düsseldorf)
3/2015	„Emotionale Intelligenz“, iGRAD (Interdisciplinary Graduate and Research Academy Düsseldorf)
3/2015	"Gentechnische Arbeiten in gentechnischen Anlagen", staatlich anerkannte Fortbildung nach §15 Abs. 2 S. 1 Nr. 3 GenTSV, iGRAD (Interdisciplinary Graduate and Research Academy Düsseldorf)
2/2015	„Führungsqualifikationen“, diskurs Strackbein GmbH via iGRAD (Interdisciplinary Graduate and Research Academy Düsseldorf)
2/2013	„Von der Idee zum Projekt. Finanzierung von Forschungsprojekten durch Drittmittel“, HHU Düsseldorf Abteilung „Forschungsmanagement“
11/2012	“Einführung in die gute wissenschaftliche Praxis”, iGRAD (Interdisciplinary Graduate and Research Academy Düsseldorf)
9/2012	„Statistik für Wissenschaftler in der Medizin (SPSS)“, medRSD (Medical Research School Düsseldorf)
3/2011	Tierversuchsschein/ Fachkundenachweis gemäß §9 TierSchG

3. Statement

Hiermit versichere ich, Sarah Jean Böddeker, an Eides Statt, dass die Dissertation von mir selbstständig verfasst und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.
Die Dissertation wurde bei keiner anderen Universität bzw. Fakultät in der vorgelegten oder einer ähnlichen Form eingereicht. Weiterhin wurden keinerlei vorherige Promotionsversuche unternommen.

Düsseldorf, den

4. Abbreviations

A, C, G, T	adenine, cytosine, guanine, thymine
α	alpha
ab	antibody
AIDS	aquired immunodeficiency syndrome
ART	assisted reproductive technology
β	beta
Bad	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphate
cIAP	cellular IAP
CS	chondroitin sulfate
CT	cytotrophoblast
CXCL1	chemokine (C-X-C motif) ligand 1
CXCR4	C-X-C chemokine receptor type 4
Da	Dalton
d	decidualized
DMEM	Dulbecco's Modified Eagle medium
DNA	deoxyribonuvleic acid
E_2	estradiol
EEC	endometrial epithelial cell
e.g.	*exempli gratia*
ESC	endometrial stromal cell
et al.	*et alii*
etc.	*et ceterea*
F	anti-Fas ab
FBS	fetal bovine serum
FCS	fetal calf serum
FoxO	forkhead box protein O
g	gramm
γ	gamma
h	hour
HELLP	hemolysis, elevated liver enzymes and low platelet count
HS	heparan sulfate
HtrA2	high temperature requirement protein A2
HUVEC	human umbilical vein endothelial cells
IAP	inhibitor of apoptosis
IUGR	intrauterine growth restriction
IITT	IL-1β, IFN-γ, TNF-α, TGF-β1
IVF	*in vitro* fertilization
JNK	c-jun n-terminal kinases
IL	interleukin
IFN	interferone
k	kilo
kd	knock down
KdS1	St-T1 with stable Sdc-1 kd
ko	knock out

l	liter
L	ligand
LH	lutenizing hormone
M	molarity
m	milli
µ	micro
MAPK	mitogen-activated protein kinase
min	minute
MMP	matrix metalloproteinases
MPA	medroxypogesterone acetate
n	nano
NCBI	National Center for Biotechnology Information
NFκB	nuclear factor kappa B
p	protein
P_4	progesterone
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol 3,4,5 trisphosphate
R	receptor
RL95-2	epithelioid endometrial carcinoma cell line
RLSdc1kd	RL95-2 with stable Sdc-1 kd
RNA	ribonucleic acid
rpm	rounds per minute
rt	room temperature
s	second
Sdc-1	Syndecan-1
sh	short hairpin
SMAC	second mitochondria-derived activator of caspases
ST	syncytiotrophoblast
St-T1	immortalized endometrial stromal cell line
tet	tetracycline
TNF	tumor necrosis factor
TGF	transforming growth factor
TRAIL	TNF-related apoptosis-inducing ligand
U	unit
XIAP	x-linked IAP
Zeo	Zeocin