Molecular basis of the embryo-maternal crosstalk in the female reproductive tract

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Table of contents

1. Introduction 9

1.1 The female reproductive tract 9

1.2 Menstrual cycle and hormonal regulation of fallopian tube and endometrium 13

1.3 Ovulation, fertilization, embryo passage, embryo-maternal dialogue and implantation 15

1.4 Aim of the studies 28

1.5 References 29

2. Articles 35

2.1 Interleukin-1 system in the human fallopian tube - No spatial but a temporal regulation of mRNA and protein expression (Molecular and cellular Endocrinolgy 303 (2009), 7-12) 35

2.2 Cathepsin S system at the feto-maternal interface (Reproduction 139 (2010), 741-748) 45

2.3 CXCL1 expression in human decidua *in vitro* is mediated via the MAPK signalling cascade (submitted to Journal of Clinical Endocrinology and Metabolism – under review) 57

2.4 Syndecan-1 *knock-down* in decidualized human endometrial stromal cells leads to significant changes in cytokine and angiogenic factor expression patterns (submitted to Reproductive Biology and Endocrinology - under review) 82

2.5 *In-vitro* culture does not alter the expression of vascular endothelial growth factor and its receptors in single murine preimplantation embryos (accepted for publication in Gynecologic and obstetric investigation) 122

2.6 Expression of the vascular growth factor receptor Neuropilin-1 in the human endometrium (Journal of Reproductive Immunology 79 (2009) 129-136) 148

2.7 Expression of vascular endothelial growth factor receptor Neuropilin-1 at the human fetal-maternal interface (in resubmission to European Journal of Obstetrics, Gynecology and Reproductive Biology) 160

3. Summary 181

4. Scientific *Curriculum Vitae* 183

5. Acknowledgement 189

6. Statement 191

Abbreviations

A, mA	ampère, milliampère (SI unit for intensity of current)
AA	acrylamide (IUPAC name 2-propenamide; formula C_3H_5NO)
AC	accession number
A, C, G, T, U	adenine, cytosine, guanine, thymine, uracil
α	*alpha*
Amp.	ampicillin (beta-lactam antibiotic; formula $C_{16}H_{18}N_3O_4S$)
A_{nm}	absorbance with a distinct wavelength [nm]
APS	ammonium persulfate (oxidizing agent, radical initiator; formula $(NH_4)_2S_2O_8$)
AA	amino acid
ART	Assisted Reproductive Technology
ASRM	American Society of Reproductive Medicine
ATCC	american type culture collection
ATP	adenosine triphosphate
β	*beta*
BLAST	basic local alignment search tool (database using Smith-Waterman algorithm)
BMP4	bone morphogenetic protein 4
bp, kbp	basepair, kilo basepair
BSA	bovine serum albumine
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
Carb.	carbenicillin (formula $C_{17}H_{18}N_2O_6S$)
Cox	cyclooxygenase
Da, kDa	dalton, kilo dalton
ddNTP	2´,3´-dideoxynucleoside 5´-triphosphate

DEPC	diethylpyrocarbonate
dES	decidualized endometrial stroma cells
DMEM	Dulbecco´s Modified Eagle medium
DMSO	dimethyl sulfoxide (formula $C_6H_{10}O_5$)
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleosidetriphosphate
DTT	dithiothreitol (Cleland´s reagent, formula $C_4H_{10}O_2S_2$)
E_2	estradiol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid (formula $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$)
e.g.	*exempli gratia*
ER	endoplasmatic reticulum
ERK	extracellular regulated kinase
ES	endometrial stroma cell
ESHRE	European Society of Human Reproduction and Embryology
et al.	*et alii*
etc.	*et cetera,* and so forth
FCS	fetal calf serum
FSH	follicle stimulating hormone
g, kg, mg, µg, ng, pg, fg	gramm, kilo-, milli-, micro-, nano-, piko-, femto-gramm
GnRH	gonadotropin-releasing hormone
GTP	guanosine triphosphate
h	hour
HRP	horse-radish peroxidase

HSA	human serum albumine
Ig	immuno globuline
LH	luteinizing hormone
IL	interleukin
IL-R	IL-receptor
KdS1	*knock-down* Syndecan-1 (St-T1 cells stably transfected with *sh*RNA for Syndecan-1)
l, ml, µl	liter, milli-, microliter
LB	luria bertani broth (lysogeny broth)
M, mM, µM	molarity, milli-, micro-molarity
MCS	multiple cloning site (polylinker)
Mg^{2+}	magnesia
min	minute
M_r	relative molecular mass
mRNA	messenger RNA
MW	molecular weight [g/mol]
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor-kappa B
nm	nanometer
OCT4	Octamer binding transcription factor
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered salt solution
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	*pondus hydrogenii*
Pos.	position

P_4	progesterone
RNA, mRNA, rRNA	ribonucleic acid, messenger RNA, ribosomal RNA
RNAi	RNA interference
RNase	ribonuclease
rpm	rounds per minute
rt	room temperature
RT	reverse transcription
s	second
Sdc	Syndecan
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gelelectrophoresis
SGI	Society of Gynecologic Investigation
shRNA	short hairpin RNA
SOX2	sex determining region Y-box 2
St-T1	SV-40 T-large antigen immortalized endometrial stroma cell line
Sdc-1	Syndecan-1
TAE	tris-acetic acid-EDTA buffer
TB	terrific broth (enriched media for bacterial growth)
TBE	tris-boric acid-EDTA buffer
TE	tris-EDTA buffer
TEMED	N, N, N´, N´-tetramethylethylenediamine (catalyzes the polymerization of acrylamide)
Temp.	temperature [°C]
T_m	melting temperature for hybridization of nucleic acids
TM	transmembrane domain
Tris	trishydroxymethylaminomethane
Tween	polyoxyethylene derivatice of sorbitan monolaurate

U	unit (rate for enzymatic activity)
uNK	uterine natural killer cell
UV	ultraviolet light with a wavelength ≤ 380 nm
V	volt (unit of clamping)
Vol.	volume
v/v	volume per volume
W	watt (unit of performance)
WHO	world health organization
w/v	weight per volume
Zeo	zeocin (formula $C_{55}H_{83}N_{19}O_{21}S_2Cu$)

1. Introduction

Reproduction is an important feature of life. Until today, the molecular basis of embryonic preimplantation development on its passage through the oviduct into the uterus and subsequent implantation into a receptive maternal endometrium to successfully establish a pregnancy is not well elucidated. International scientific societies as the European Society of Human Reproduction and Embryology (ESHRE), the American Society of Reproductive Medicine (ASRM) and the Society of Gynaecologic Investigation (SGI) deal with human fertility and embryology supporting experimental and practical research and deciding clinical practice guidelines in the field of the human reproductive tract and the treatment of infertility applying assisted reproductive technology (ART).

General aspects regarding basic processes of the female cycle, fertility and early embryonic development will be explained further in the following paragraphs.

1.1 The female reproductive tract

In human, the sexual development starts at the 6th week of gestation. The proximal oviduct, the uterus and the vagina are of paramesonephric origin (*ductus muellerius*). The *ductus mesonephricus* (*ductus wolfius*) also takes part in the development of the vagina. The cranial parts of the *ductus muellerius* differentiate into the fallopian tubes. The ovaries originate from epithelial coelom and primordial germ cells. Within the 5th and 6th week of gestation, primordial germ cells migrate into the indifferent gonadal segment. These cells initiate mitosis and form gametocytes (about 6×10^6 gametocytes starting in the 12th week of gestation). These primary oocytes arrest between meiotic pro- and metaphase until the age of puberty [1]. During fetal development, the uterus changes in shape and growth due to caudal growth of the uterovaginal primordium, rostral extension of the fused portion of the *paramesonephric* ducts, and a proliferation and thickening of the *mesenchyme*. By midgestation, the cadual 2/3 of the uterus becomes the cervix, and the cranial 1/3 is bulbous and becomes the corpus [2,3].

Some layering of the mucosa is evident at this time, and by 20th week of gestation smooth muscle cells develop in the muscular layer (*myometrium*), growing during the second half of gestation and resulting in the pear-like shaped uterus as seen in adults (Figure 1) [4]. Before the 20th week of gestation, the uterine cavity of the human fetus is lined by a single layer of columnar epithelium without glands. At mid-gestation, the epithelial lining of the cavity forms pouches of cells that grow into the underlying *mesenchyme*, which become glands by the 7th month of pregnancy. At this time, the endometrium responds to the high levels of steroid hormones from the placenta. By the 9th month of gestation, the fetal endometrium is hypertrophic, with an oedematous stroma, blood vessels and actively secreting, coiled glands. In the newborn, the endometrium has features of proliferative and secretory endometrium [4,5]. During infancy and childhood the uterus grows consistent with overall body growth. The glands elongate and reach the basal portion of the endometrium. After puberty, uterine (endometrial and myometrial) growth is stimulated by circulating estradiol (E_2) and progesterone (P_4), with the corpus becoming larger than the cervix, as in the adult (Figure 1).

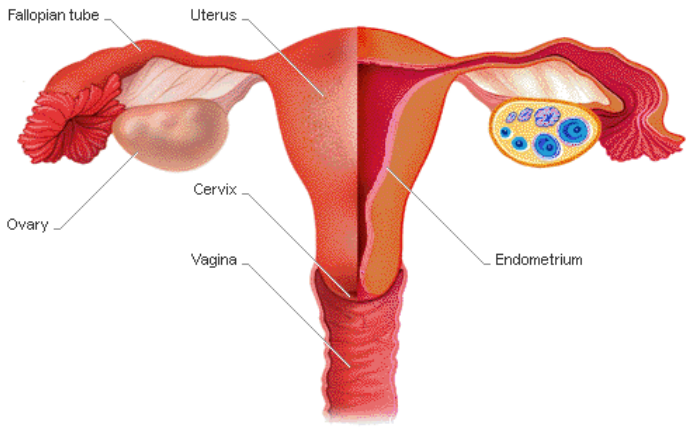

Figure 1: The adolescent female reproductive tract consisting of ovaries, fallopian tubes, uterus and vagina. (http://images.encarta.msn.com/xrefmedia/aencmed/targets/illus/ilt/T530006A.gif).

In the adult, each oviduct is about 13 cm long and is divided into four regions:

1. *intramural region* (passing through the uterine wall),
2. *isthmus* (proximal 1/3 of the tube, extending laterally from the intramural region to the ampulla),
3. *ampulla* (distal 2/3 of the oviduct, contiguous with the isthmus),
4. *infundibulum* (trumpet-shaped end of the tube, open to the peritoneal cavity by the abdominal osteum and containing multiple folds, the "*fimbriae*") [6].

The oviduct is composed of three distinct histologic layers: an inner *mucosa*, middle *muscularis*, and the outer *serosa*. The mucosa forms an elaborate system of highly branched, interconnected, longitudinal folds in the ampulla, resembling a labyrinth, with less branching and folding along the tube towards the isthmus [6]. A single columnar epithelium lines the mucosa and is comprised of ciliated cells and non-ciliated secretory cells. The *lamina propria* provides the support for the mucosa and is comprised of a thin vascular layer of loose connective tissue. The muscularis is comprised of an inner circular layer and an outer, longitudinal layer of smooth muscle and connective tissue. The serosa is the outer layer [6].

The uterus is divided into three anatomical parts:

1. *corpus* (body - containing the *cavum uteri*),
2. *fundus* (top portion),
3. *isthmus* (which is contiguous with the cervix).

The uterine corpus is composed of three histological layers: the inner endometrium, the muscular myometrium and the outer perimetrium or serosa adjacent to the *peritoneum*.

The endometrium lining the corpus is most profoundly affected by changes in circulating steroid hormones E_2 and P_4.

The endometrium has a complex cellular composition, including simple columnar epithelium (glandular and luminal) of secretory and ciliated cells, stromal fibroblasts, vascular endothelium and smooth muscle, and immune cells [7]. The layer of columnar epithelium, "luminal epithelium" is the interface between the uterine cavity and the endometrial mucosa.

Glandular epithelium lines the tubular or branched glands that can extend as deep as the endometrial-myometrial junction. The stromal compartment (*lamina propria*) is comprised of highly cellular connective tissue with an extracellular matrix that contains few connective tissue fibers and resembles embryonic mesenchyme. The endometrial-myometrial junction is indistinct, without an intervening submucosal membrane. Parts of the basal endometrium extend in the proximate regions of adjacent myometrium [8].

The endometrium is comprised of four histologically defined zones [9]. These compartments are:

1. zone I, comprised of luminal epithelia and sub-adjacent, densely packed stroma;
2. zone II, the upper endometrium in which the straight region of the glands course and are widely separated by stroma;
3. zone III, mid-regions of the glands, widely separated by stroma;
4. zone IV, the deepest portion of the glands in a fibrous stroma, adjacent to the endometrial-myometrial junction.

Zones I and II comprise the *functionalis* layer, and zones III and IV comprise the *basalis* layer [2]. In humans, the *functionalis* responds cyclically to ovarian steroid hormones, is the site of embryonic implantation, and is shed with the menstrual bleeding in the absence of implantation. Cells of the *basalis* including stem cells participate in regeneration of the tissue after the menstrual bleeding [3].

The onset of puberty is associated with high hypothalamic gonadotropin-releasing hormone (GnRH) pulsing responsible for the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from anterior pituitary gland resulting in ovarian activation (Figure 2). Leptin, a protein hormone which is responsible for nutrition intake and metabolism, is associated with the initial GnRH pulsing [10]. The GnRH pulse activity is critical for successful reproductive function until menopause.

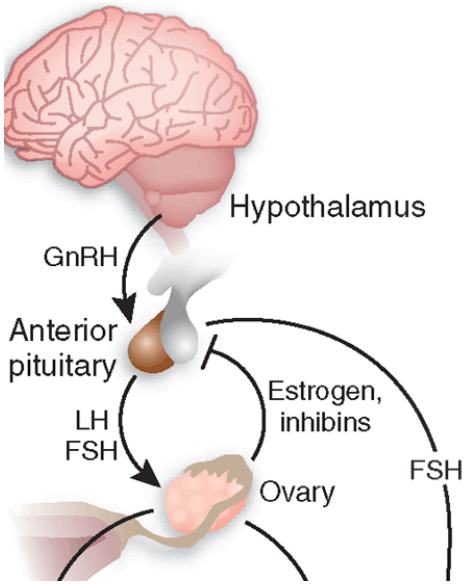

Figure 2: Overview of GnRH pulsing and feedback loop (http://www.nature.com/nm/journal/v12/n6/images/nm0606-612-F1.gif).

Fallopian tubes and endometrium are highly sensitive to changes in circulating ovarian and adrenal cortex derived, menstrual cycle dependent steroid hormones E_2 and P_4 critically determining the menstrual cycle.

1.2 Menstrual cycle and hormonal regulation of fallopian tube and endometrium

The human endometrium underlies morphological and structural changes during the menstrual cycle in preparation for embryo implantation or menstrual shedding in absence of implantation, respectively [11] (Figure 3).

The E_2-dominated proliferative phase of the endometrium, which lasts from the end of menstrual bleeding until ovulation, is characterized by proliferation of the *lamina functionalis*, endometrial glands and endometrial stromal tissue as well as angiogenesis of endometrial blood vessels. In the oviduct, remarkable changes occur in epithelial ciliogenesis and secretory cell development [12].

The P_4-dominated secretory phase, which starts at ovulation with the peak of LH and ends with the following menstrual bleeding, is accompanied by high secretory activity of

endometrial glands, elongation and growth of spiral arteries and decidualization and re-organization of endometrial stroma enabling embryonic invasion during the window of implantation between day LH peak +8 until LH+10 [13]. Growth factors and cytokines are involved in paracrine and autocrine actions to affect the histologic and biochemical signatures of the endometrial *functionalis* [7]. Under the influence of P_4, the oviductal epithelium atrophies, regresses, and becomes quiescent, primarily through cellular apoptosis.

Regarding the establishment of pregnancy, all of these changes - endometrial re-organization via matrix metalloproteinases (MMP) and further matrix modifying enzymes, angiogenesis, influx of immunocompetent cells (e.g. specialized uterine natural killer cells (uNK)), biochemical changes, cleavage and maturation of the embryo after fertilization during its passage through the fallopian tube - need to be synchronized on molecular level in a coordinated embryo-maternal dialogue facilitating embryonic invasion and implantation. The knowledge about the factors involved in these early processes on embryo and maternal side increased in the last decades supported by extensive DNA and protein microarray analyses [14-16].

In the absence of implantation, the endometrium undergoes apoptosis, tissue desquamation followed by menstrual bleeding, and regeneration ensured by mechanisms likely involving stem cells in the *basalis* region of the tissue [17].

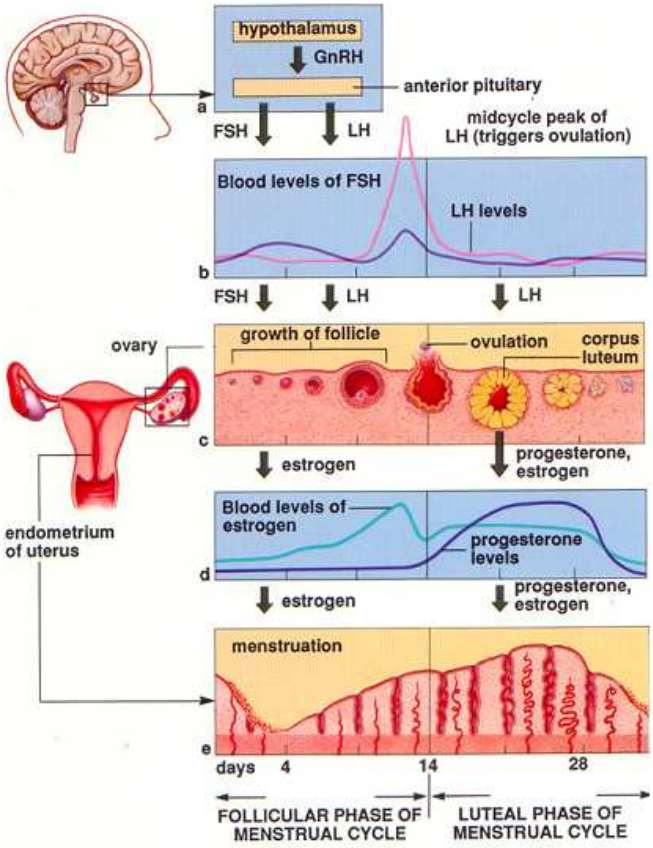

Figure 3: Overview of the female menstrual cycle. a) hypothalamic GnRH release, b) subsequent FSH and LH levels in peripheral blood, c) leading to growth of follicles and ovulation, d) ovary (and adrenal cortex) derived blood levels of E_2 and P_4 throughout the cycle, e) endometrial lining (http://www.soc.ucsb.edu/sexinfo/images/05-07-Menstrual.jpg).

1.3 Ovulation, fertilization, embryo passage, embryo-maternal dialogue and implantation

The process of ovulation is characterized by biochemical and morphological changes finally leading to the release of a mature oocyte and the transformation of the follicle into the *corpus luteum*. Generally, an ovarian follicle consists of different, interacting layers of cells surrounding the developing primary oocyte. Granulosa cells encompass the oocyte and proliferate in response to circulating gonadotrophins. During the E_2-dominated proliferation,

respectively, FSH stimulates the sythesis of LH receptors on granulosa cells. Granulosa cells respond to elevating LH levels by an increase of cyclicAMP (cAMP) and production of P_4. The granulosa cells are surrounded by a small extracellular matrix and outside of this membrane, the *theca interna* and *externa* are found. As described before, primary oocytes arrest between meiotic pro- and metaphase until the age of puberty. For each primary oocyte that undergoes meiosis, only one functional oocyte is produced. The mature oocyte is arrested in this stage until fertilization and meiosis is accomplished after the invasion of a sperm. In general, due to the so-called dominance only one mature oocyte is produced per menstrual cycle. Polar bodies are the by-products of the oocyte´s division during meiosis. The haploid polar bodies - the first polar body is haploid with 23 duplicated chromosomes, the second is haploid with 23 single chromatides - are the by-products of this division, and are essentially extruded by the oocyte. By analyzing the polar bodies, it is possible to infer the genetic status of the mother´s contribution to the embryo [18].

Different non-steroidal mechanisms influence the development of the mature oocyte, for example the meiotic arrest and the temporal pattern of luteinization of the granulosa cells [19,20]. The LH peak determines the ovulation, as the release of the mature ooycte from the follicle. The follicle first forms a *corpus hemorrhagicum* before it becomes a *corpus luteum*. While the oocyte traverses the fallopian tube on its way into the uterus, the *corpus luteum* remains in the ovary. The *corpus luteum* is essential for maintaining pregnancy in females by producing E_2 and P_4 supporting the decidualization of the endometrium and its maintenance. In the absence of fertilization of the mature ooycte, the corpus luteum degenerates after 14 days of P_4 production (*corpus albicans*) [21] (Figure 4).

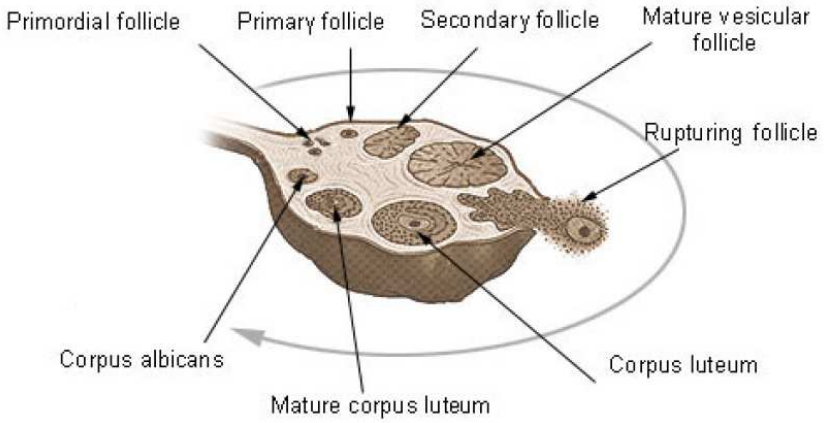

Figure 4: Follicular fate during menstrual cycle - from recruitment to selection, dominance and degeneration (http://upload.wikimedia.org/wikipedia/commons/0/05/Folliclesinovary.jpg).

The physiology of the fallopian tube has a great impact on sperm transport to the mature oocyte and *vice versa* on embryo transport lasting 3-4 days from the ampulla/isthmic region of the tube, where fertilization occurs, to the receptive endometrium of the uterus [22].

At the beginning, the number of spermatozoa reaching the site of fertilization is enormously reduced from more than 150 × 10^6 / ml to less than 1000 spermatozoa in the ampulla [23]. The high number of non-fertilizing spermatozoa is supposed to influence the reproductive process. Male spermatozoa need to render competent in order to fertilize during the passage through the uterine cavity by capacitation which is influenced by oviductal tissue [24]. During capacitation, the acrosomal membrane of the spermatozoon gets destabilized by modification of steroid and glycoprotein content resulting in a more fluid membrane with increased calcium (Ca^{2+}) permeability [25]. Ca^{2+} influx increases sperm mobility. A male tripeptide, the fertilization promoting factor, produced in male prostate gland and released to seminal fluid, supports capacitation. Binding to its receptor T-complex protein 11 activates intracellular adenylyl cyclase activity and cAMP synthesis [26] (Figure 5).

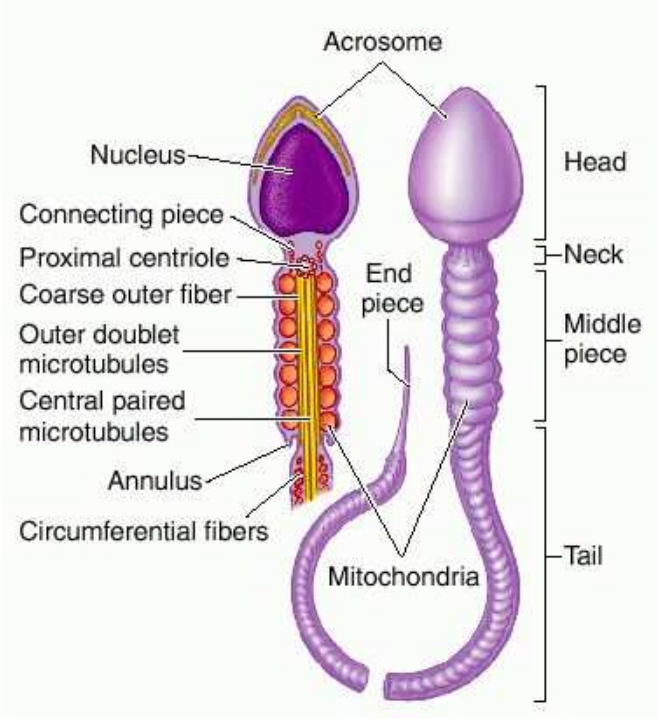

Figure 5: Structure of a male spermatozoon highlighting important structures for oocyte invasion of head including nucleus, neck and middle piece (www.medical.dictionary.thefreedictionary.com/_/viewer.aspx?path=dorland&name=spermatozoon.jpg).

Upon ovulation, the mature follicle ruptures and the *cumulus oophorus* is extruded from the ovary and gets in contact with the fimbriae of the *infundibulum* [27]. The cilias´ action during muscle contractility, which could be pharmacologically inhibited in rats, moves the oocyte back- and forward during its transport through the ampullary part of the fallopian tube [28]. When the oocyte reaches the ampullary/isthmic region the transport stops for possible fertilization in the presence of competent spermatozoa. Oviductin mRNA was detected in human tubes in periovulatory period and was assumed to support fertilization and early embryo development [29]. Before penetration of the oocyte takes place, the spermatozoon needs to be attracted via chemotaxis. Cumulus-cell derived P_4 acts as a key player in human sperm chemotaxis towards the oocyte leading to activation of variant signalling cascades including increase of intrasperm cAMP level [30]. Preceding penetration, the head of the sperm, which contains the nucleus, undergoes modifications in acrosome reaction when it approaches the *zona pellucida* of the oocyte. The acrosome and the plasma membrane of

the sperm fuse. This fusion results in releasing acrosomal enzymes, for example hyaluronidase, acrosin, acid phosphatase, β-glucuronidase, β-glucosidase, β-N-acetylglucosaminidase, β-galactosidase and β-N-acetylgalactosaminidase and interaction with the *zona pellucida* [31]. As a result, granula inside the oocyte fuse with the outer membrane and block further penetration. ZP3, an oocyte glycoprotein, interacts with its receptor on sperm acrosome β1,4-galactosyl transferase receptor [32]. This reaction is species specific and inhibits penetration of oocytes with foreign sperm. Parts of the sperm membrane fuse with the oocyte membrane and the head of the sperm fuses with the oocyte. Upon penetration the oocyte is called activated and undergoes the second part of the meiosis leading to the haploid phenotype. Haploid nuclei of sperm and oocyte can fuse to form the zygote. The ongoing transit of the zygote inside a bolus of fluid through the isthmic region of the fallopian tube is characterized by strong contractility of the myosalpinx. The impulse of cilia beating was found to be influenced by prostaglandins, adenosinetriphosphate (ATP) and platelet-activating factor *in vitro* in physiologic concentrations [33]. The time of transport of the oocyte and zygote is species-specific, for example in women the zygote remains in the ampulla for a relatively long time, whereas a rodent zygote passes the ampulla in very short time. Therefore, this time-regulated transport associated with the modifications on molecular level in the reproductive tract and interaction with the embryo supports a regular embryonic implantation into the receptive decidualized endometrium [22].

Embryogenesis starts with the fertilization of an oocyte including subsequent cleavages, maturation, implantation and organogenesis and lasts about 9 weeks until foetus stage. Before fusion of maternal and paternal genome, the pronuclei stage appears that is observed in IVF techniques about 18h after fertilization. After fusion of the parentals´ genomes, the zygote undergoes cleavages (mitotic divisions) and differentiations during the transit through the fallopian tube without increasing in size being surrounded by the *zona pellucida*. Embryonic cells are totipotent up to 8-cell stage [34]. Totipotency is the ability of a cell forming a complete organism with extraembryonic tissue (placenta). After that stage, cellular fate is determined. Even the zygote is morphologically asymmetric consisting of an animal pole -

the future ectoderm and mesoderm of the germ layers - and a vegetal pole forming the future endoderm. The first cleavage always occurs along the vegetal-animal axis of the zygote. The second cleavage is perpendicular to the first. Following cleavages occur rotationally. The formation of the dorsoventral axis is controlled by the maternal genome (Figure 7). A dorsoventral patterning defect was shown in zebrafish embryos lacking maternal and zygotic POU domain transcription factor POU2 - an ortholog of mammalian Octamer binding transcription factor 4 (Oct4)/ POU class 5 homeobox 1 (Pou5f1) [35]. Oct4 functions as a marker of undifferentiated cells and pluripotent cells. Therefore, cells with a pluripotent ability could be identified in human germ cell tumors [36]. The cells of the embryo are called blastomeres up to blastocyst stage. Up to 4-cell stage, the maternal genome determines embryonic development, between 4- and 8-cell-stage the embryonic genome is activated [37].

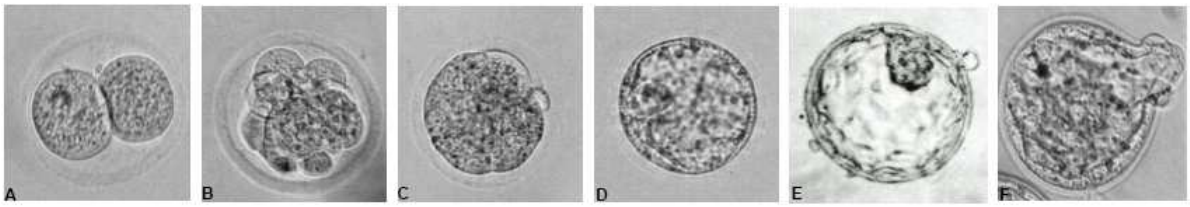

Figure 6: Mammalian embryonic development from 2-cell stage (A), 8-cell stage (B), compacted morula (C), early blastocyst (D), late blastocyst (E) to hatching blastocyst (F) (modified after Krüssel *et al.* [38]).

Four cleavages occur up to the morula stage when 16 cells form a dense ball-like unit (Figure 6). Soon, cells arrange into an outer layer called trophoblast and an inner layer developing into the future embryo. Trophoblast and inner layer or inner cell mass remain in contact only at the embryonic pole of the blastocyst (Figure 6). Fluid collects between the trophoblast and the inner cell-mass filling the blastocyst cavity. Cells of the trophoblast first provide nutrition to the embryo and subsequent develop into placental tissue. Prior to gastrulation, cells of the trophoblast differentiate into cytotrophoblast as the inner cell layer and the syncytiotrophoblast as a thick cell layer. Cells of the syncytiotrophoblast lack cell boundaries, are invasive and grow into the maternal decidua during implantation. It was

reported that the histone H3 Lys 9 methyltransferase (Eset) is an epigenetic factor critical for the development of the inner cell mass interacting with OCT4 during morula stage. It was proposed that Eset restricts the potential of pluripotent cells of the extraembryonic trophoblast lineage [39]. The differentiation into inner cell mass and trophectoderm is completed in blastocyst stage approximately at day 5 after fertilization *in utero* (Figure 7). Pluripotent embryonic stem cells are isolated from the inner cell mass and the trophectoderm is used as well as a source for pluripotent stem cells. Recently, it was reported that inner cell mass and trophectoderm express factors regulating each other. Fibroblast growth factor receptor substrate 2 alpha mediates fibroblast growth factor 4 signalling and activates eytracellular regulated kinase (ERK) signalling cascade in trophoblastic cells and enhances expression of homeobox protein Cdx2, which is important for the formation of the placenta. Cdx2 binds to the promoter region of bone morphogenetic protein 4 (Bmp 4), another factor characterising stem cell activity [40]. Lately, overexpression of combinations of defined transcription factors localized in the inner cell mass, namely, OCT4, sex determining region Y-box 2 (SOX2) and homeobox protein NANOG resulted in inducible somatic stem cells improving regenerative medicine techniques [41] (Figure 7). These stem cells are able to generate a new organism but lack the ability to generate extraembryonic tissues.

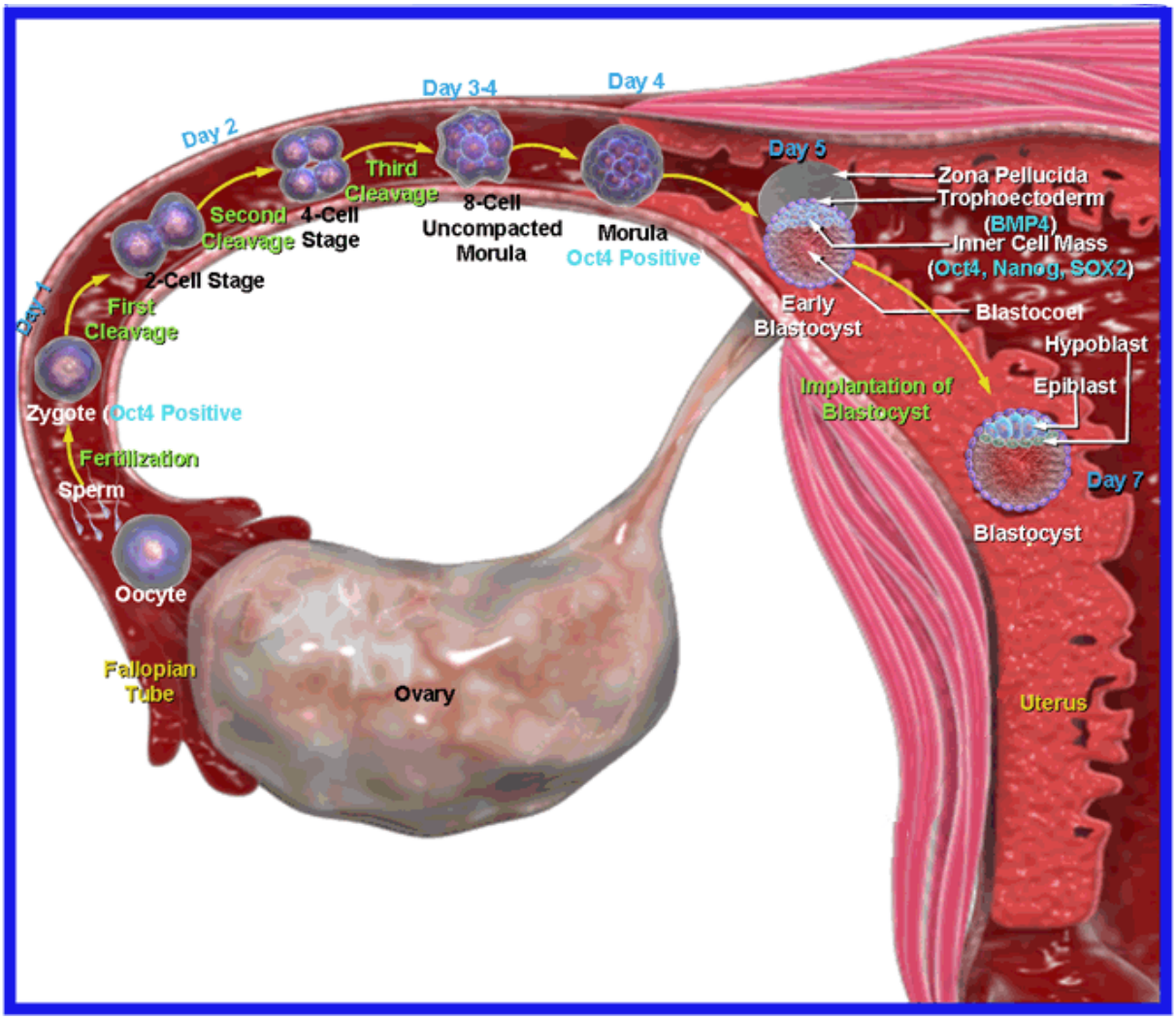

Figure 7: Embryo development from fertilization, first cell cleavages and maturation, transit through the fallopian tube to the uterus, implantation into the receptive endometrium and further development until gastrula stage on day 15. Expression of stem cell marker Oct4 already starts in the zygote. BMP4, Nanog and SOX2 were shown to be expressed during implantation stage of the hatched embryo (modified after http://www.sabiosciences.com/pathway.php?sn=Human_Early_Embryo_Development).

Prior to implantation, the embryo needs to hatch out of the *zona pellucida*. Hatching seems to be regulated by cellular dynamic, for example actin-based trophectordermal projections, and autocrine as well as paracrine molecules, for example growth factors, cytokines and proteinases. It was reported from hamster blastocysts that embryo-derived cathepsins, prostaglandins and nuclear factor-kappa B (NF-κB) signalling are involved in the process of hatching [42].

Already during the preimplantation period, while the embryo passes the fallopian tube and enters the uterus, an embryo-maternal dialogue via embryonic secretome and fallopian epithelial and decidual secreted factors occurs.

Interleukin-1 beta (IL-1ß) and its receptor IL-1 receptor type 1 (IL-R1) could be detected immunohistochemically in samples of fallopian tubes of both proliferative and secretory phase, whereas the antagonizing molecule, IL-1 receptor antagonist (IL-1ra) was expressed only in samples of secretory phase [43]. Hence, the expression of the IL-1 system was examined in human endometrium during menstrual cycle [44]. IL-1R1 was found to be expressed in glandular cells and IL-1ß in endothelial cells of spiral vessels and stromal cells of the human endometrium with an increase from proliferative to secretory phase [44]. Face to face with the endometrium, IL-1R1 mRNA was detected in blastomeres from 12 normal, dipronuclear zygotes. 75 % of the embryos examined expressed IL-1ß mRNA and only 17 % the antagonist IL-1ra. The embryos expressing IL-1ra mRNA arrested in development before reaching blastocyst stage [45]. The IL-1 system connects embryo development, endometrial receptivity and immune system, which bears an important impact on the successful implantation of the semi-allograft embryo [46]. In the last decade, many studies focused on the identification of immune competent and specialized cells, for example uterine natural killer cells, at the embryo-maternal interface during proper and failed embryonic implantation [47].

Another family with an immunocompetent impact are chemokines that are small proteins with conserved disulphide bridges guiding the chemotactic migration of cells, for example lymphocytes or neutrophils. Moreover, chemokines with a glutamic acid - leucin - arginine (ELR) motif before the first cysteine in the amino acid sequence feature angiogenic abilities, for example chemokine ligand 1 (CXCL1) or CXCL8 [48,49]. CXCL1 mRNA and protein was increased in endometrial stroma cells of secretory phase being regulated by steroid hormones and infection-associated molecules, for example lipopolysaccharide and IL-1ß [50]. Moreover, CXCL1 was identified as the most upregulated gene in decidualized endometrial stroma cells after incubation with trophoblast-conditioned media [14]. The corresponding receptor, CXCR2, was found to be expressed in the intermediate cell mass of zebrafish

embryos at day 10 after fertilization underlining the hypothesis of an embryo maternal interaction via chemokines [51]. A further family of molecules that takes part in embryo-maternal interaction is the vascular endothelial growth factor (VEGF) family. Angiogenesis as the growth and development from blood vessels from pre-existing ones is a key process during endometrial preparation for pregnancy and implantation. The mRNAs of VEGF receptors (VEGFR1 and VEGFR2, formerly FMS-like tyrosine kinase (Flt1) and kinase insert domain receptor (KDR)) and the soluble sVEGFR1 were detected in human epithelial and stroma cells of cycling endometrium with constant levels of VEGFR1 and VEGFR2. The mRNA expression of the antagonizing sVEGFR1 decreased during secretory phase possibly sensitizing the maternal endometrium for embryonic angiogenic stimuli [52]. The mRNAs for the different splicing variants of $VEGF_{121-206}$ were also detected in human preimplantation embryos supporting the idea of an angiogenic interaction between maternal endometrium and embryo [53,54].

Especially in IVF techniques when the development of the embryo takes place extra corporally, intense research effort is undergone to compare *in vivo* and *in vitro* development as well as development in different commercial media for modifications of the embryonic mRNA expression. Due to ethical reasons and legal restrictions, most studies are performed in animal models, for example in mouse, bovine and ruminent embryos [55]. Particularly, studies from bovine embryos supporting cattle breeding reveal interesting aspects regarding maturation, somatic cell nuclear transfer, *in vitro* culture and embryonic cloning during blastocyst stage [56-58]. The importance of a proper dialogue between embryo and mother is highlighted in high rates of pregnancy failure after transfer of *in vitro* cultured embryos derived from somatic cell nuclear transfer [59]. Therefore, the mRNA profile of endometrial samples at day 18 of pregnancy was examined on a cDNA microarray enriched for transcripts differentially expressed in the endometrium and oviduct epithelium during the E_2 cycle and early pregnancy after day 7 blastocyst transfer of embryos derived from somatic cell nuclear transfer and common *in vitro* fertilization. A total of 58 transcripts were different and mRNA levels of orphan nuclear receptor COUP-TFII (NR2F2) and gap junction protein

connexin 43, both were shown to be involved in uterine receptivity and placentation before, were significantly decreased in endometrial biopsies after somatic cell nuclear transfer [60,61]. This seems to be an obvious link between embryonic origin and development and maternal endometrial receptivity [59].

Early interactions between maternal decidua and developing embryo seem to be involved in embryonic “homing” into the receptive endometrium. Embryonic “homing” was compared to leukocyte trafficking as similar adhesion molecules, for example integrins and L-selectin, as well as chemokines are involved in apposition, attachment, invasion and subsequent implantation. Both - embryo and leukocytes - need to pass epithelia in order to enter the endometrial stroma [62,63]. During invasion the embryonic trophoblast turns invasive and invades the stroma up to the maternal vessels by expression of matrixmetalloproteinases (MMP) 2 and 9 [64]. Endometrial expression of chemokines, CXCL8 and chemokine ligand 5 (CCL5, formerly RANTES), is meant to induce activation of endometrial adhesion molecules during the window of implantation with corresponding receptors on the surface of the blastocyst [65,66]. Furthermore, CXCL1 showed the most upregulated mRNA expression in decidualized endometrial stroma cells co-incubated with trophoblast-conditioned media [14]. Migration of human trophoblast cells is directed *in vitro* by CXCL10, IL-6, insulin-like growth factor-II (IGF-II) and insulin-like growth factor binding protein 1 (IGFBP1) [67-69]. In summary, the key processes of embryonic invasion at the embryo-maternal interface are regulation of trophoblast invasiveness, proliferation and differentiation mediated for example by MMPs and their inhibitors tissue inhibitor of MMP (TIMP-1 and -3), chemokines, proteinases and angiogenic factors expressed by maternal as well as embryonic tissue, remodelling of spiral arteries during early placentation and maternal acceptance of the semi-allograft embryo (Figure 8) [70-76].

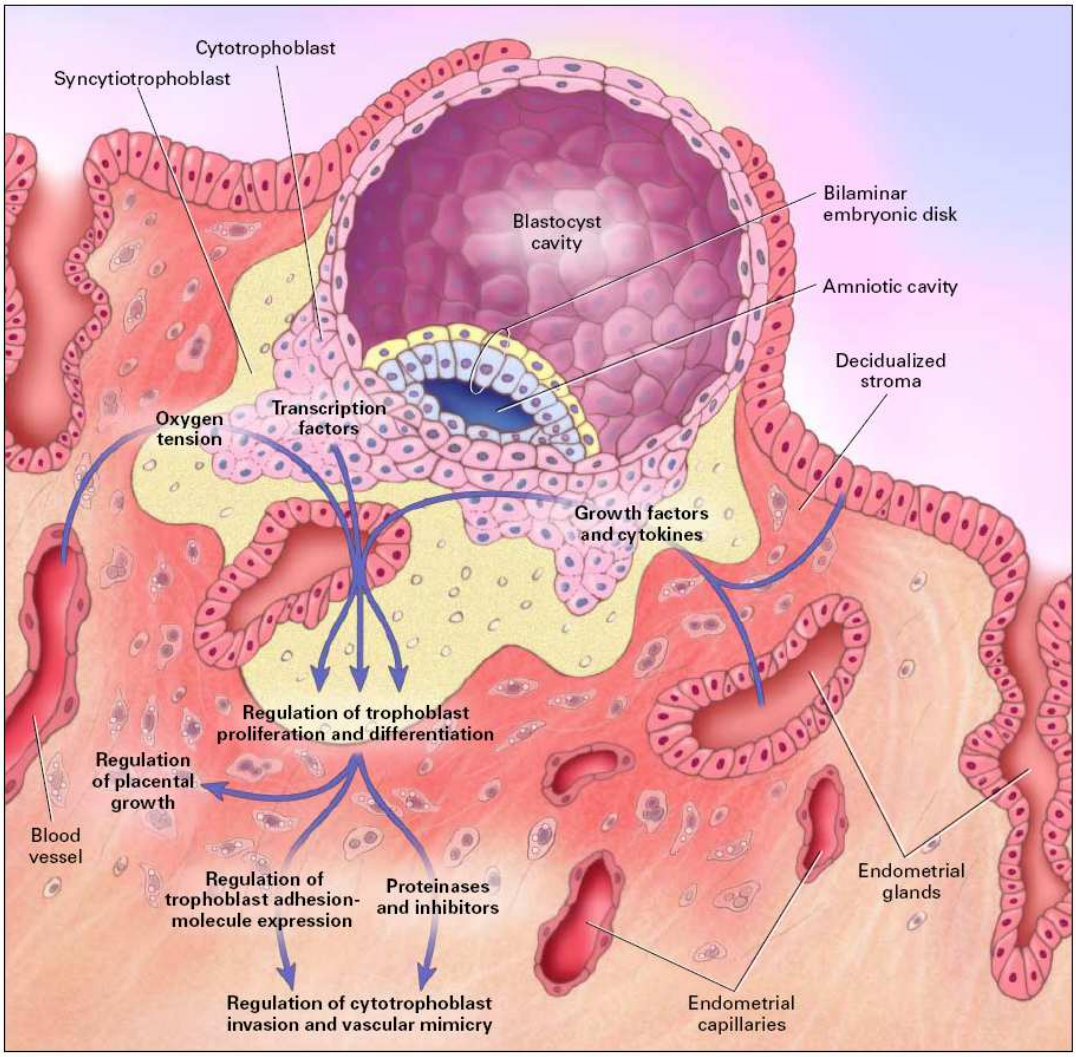

Figure 8: Overview of an invading blastocyst at day 9/10 after conception and the processes necessary for invasion at the embryo-maternal interface including regulation of trophoblast proliferation, differentiation and angiogenesis [77].

Regarding embryogenesis during implantation, the amniotic cavity is formed. The adjacent layer of the cavity is formed by the embryonic disk, followed by embryonic ectoderm and entoderm (Figure 8). The following steps are characterized by extensive cell migrations and invasions. The primitive knot (Hensen´s node) starts as a regional knot anterior to where the outer layer of cells will begin to migrate inwards - the primitive streak. It is produced by a thickening of the axial part of the ectoderm. The cells multiply, grow downward, and intermix with those of the subjacent entoderm. From the sides of the primitive streak, a third layer of cells, the mesoderm, extends lateralward between the ectoderm and entoderm. The

blastoderm now consists of three layers: ectoderm, mesoderm, and entoderm. Each has distinctive characteristics forming a complete body:

1. ectoderm develops into skin cells, neurons and pigment cells,
2. mesoderm generates skeletal and smooth muscles, blood cells and tubulae of the kidney,
3. endoderm forms lung cells, thyroid and pancreatic cells (Figure 9) [78].

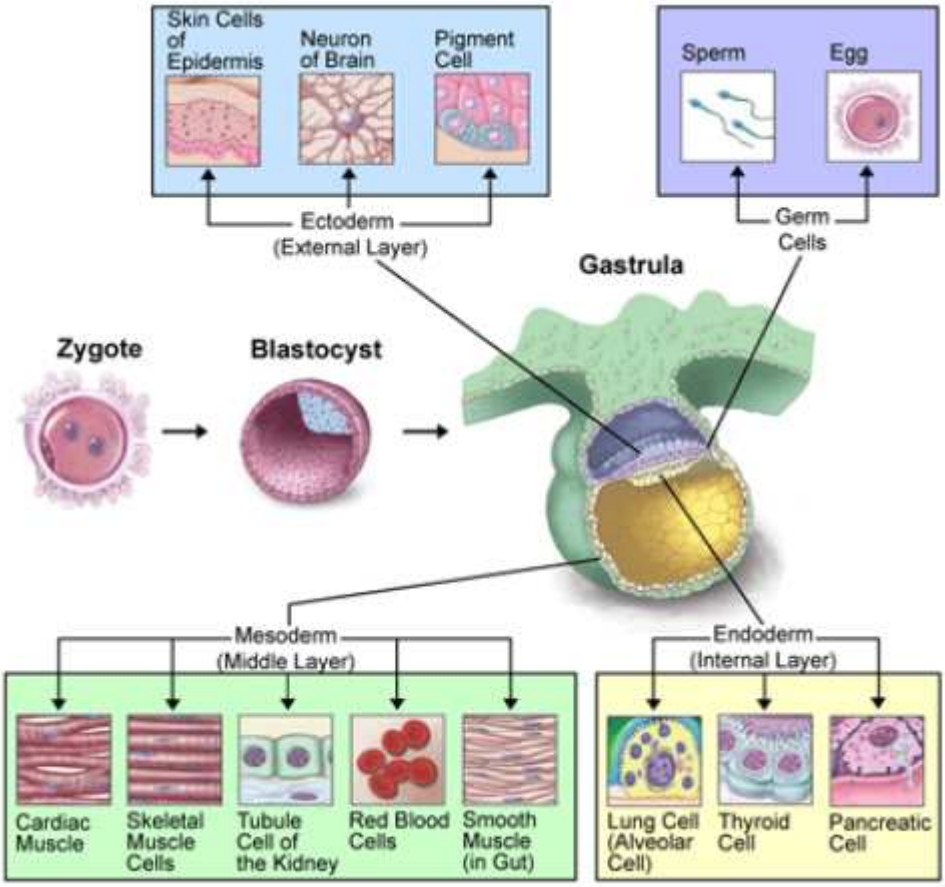

Figure 9: Development of embryonic germ layers from zygote to gastrula stage. Cells of the ectoderm develop to skin cells, neurons and pigment cells. Smooth and skeletal muscle, tubule of the kidney and erythrocytes are generated from the mesoderm. Embryonic endoderm results in lung cells, thyroid and pancreatic cells. Germ cells are separately established (http://www.ncbi.nih.gov/About/primer/genetics_cell.html this].

1.4 Aim of the studies

The studies represented in the following paragraph focussed on the characterization of the molecular processes at the embryo-maternal interface. Different aspects of expression and localization of proteinases and inhibitors, interaction of co-receptors of chemokines and growth factors and their expression were examined applying different techniques from immunohistochemistry, analysis of mRNA and protein expression and RNA interference.

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2. Articles

2.1 Interleukin-1 system in the human fallopian tube - No spatial but a temporal regulation of mRNA and protein expression (Molecular and cellular Endocrinolgy 303 (2009), 7-12)

AP Hess, DM Baston-Buest, A Schanz, J Hirchenhain, P Bielfeld, JS Kruessel

The human fallopian tube is the place of fertilization and also provides the natural environment for the first 5 days of embryonic development. It is, as well, the most common place for ectopic implantation. The preimplantation embryo produces several factors during its development to signal its presence to the maternal organism. Appropriate interaction between the preimplantation embryo and the maternal system is at least partly controlled by paracrine cytokines.

The Interleukin-1-system is composed of two agonists, Interleukin-1α (IL-1α) and Interleukin-1β (IL-1β), one antagonist, the Interleukin-1 receptor antagonist (IL-1ra) and two membrane-bound receptors, Interleukin-1 receptor type I (IL-1R1) and II (IL-1R2). Only the binding of either IL-1α or IL- β to the IL-1R1 results in signal transduction. The IL-1 system is intimately involved in embryonic implantation. There is an increasing body of evidence that the IL-1 system is involved in early embryonic development and implantation.

The aim of our study was to detect a potentially spatial and temporal IL-1β, IL-1ra- and IL-11-expression pattern on mRNA and protein-level in human fallopian tubes and to describe possible hormonally regulated changes since these changes might influence the time and place of embryonic implantation.

All 19 collected oviduct samples from the proliferative (n=8) and secretory (n = 9) phase of the cycle and postmenopausal (n=2) women were divided into fimbrial, ampullary and isthmic parts and investigated separately by RT-PCR.

The following investigation of IL-1β, IL-1ra and IL-1R1 expression showed that the presence or absence of the respective gene was constant regarding the spatial distribution.

This shows that the gene expression of IL-1β, IL-1ra and IL-1R1 within the length of the fallopian tube has no spatial difference.

All 23 collected oviduct samples from the proliferative (n=8) and secretory (n = 9) phase of the cycle and from postmenopausal (n = 2) women and extra- (n = 3) and intrauterine (n = 1) pregnancies were separately investigated by RT-PCR for temporal expression patterns of IL-1β, IL-1ra and IL-1R1. The patient with the intrauterine pregnancy received a radical hysterectomy due to cervical cancer in the 9th week of pregnancy.

IL-1R1 was expressed in all samples regardless the hormonal situation. IL-1ra mRNA could be detected in samples of the secretory phase and in intrauterine pregnancy, whereas IL-1β could be detected in proliferative and secretory phase fallopian tubes and in samples of intra- and extra-uterine pregnancy. This shows that the gene expression of IL-1β, IL-1ra and IL-1R1 is temporally regulated within the human cycle.

The protein expression of IL-1β, IL-1ra and IL-1R1 of proliferative phase versus secretory phase samples was evaluated using immunohistochemistry.

We could show a strong expression of IL-1β foremost in the luminal epithelium and also in the small vessels endothelium of secretory phase oviducts, whereas the staining for IL-1β in the luminal epithelium of proliferative phase oviducts was less intense and only a weak staining was localized in the small vessels endothelium.

The IL-1R1 was shown to be expressed in the luminal epithelium, small vessels endothelium and glandular epithelium of secretory phase samples. In comparison the proliferative sample showed only a sporadic staining for the IL-1R1 in the luminal epithelium and no staining either in the vessel endothelium or in the glandular epithelium There was an intense staining of IL-1ra in the luminal epithelium and the small vessel endothelium of secretory phase oviducts whereas a staining for IL-1ra in proliferative oviducts was completely absent revealing a strict temporal expression of IL-1ra.

The fallopian tube on the maternal side plays an important role in providing the place for fertilization as well as an active component for transport and early development of the embryo. Within the oviduct, remarkable changes occur in dependence to the sex steroids E_2

in the proliferative phase and P_4 in the post-ovulatory secretory phase indicating a hormonal cyclicity of the oviduct comparable to the endometrium.

Since we have shown before that the human embryo produces IL-1β when cultured *in vitro*, it is reasonable that it also produces IL-1β during its passage through the fallopian tube.

Consequently, it is very likely that the fallopian tube as the early communication partner for the developing embryo itself expresses components of the IL-1 family.

Furthermore, our aim was to elucidate whether the sex steroids E_2 and P_4 have an influence on the temporal expression of IL-1β, IL-1ra and IL-1R1 during the reproductive phase. Therefore, fallopian tubes from the proliferative and secretory phase of the menstrual cycle as well as oviducts from postmenopausal women were investigated. This showed that IL-1β was only present in samples from the proliferative and secretory phase but in none of the postmenopausal oviducts suggesting a regulation by sex steroids and a role in the process of human reproduction.

In contrast, IL-1ra mRNA was only detected in P_4 dominated secretory phase samples and in the intrauterine pregnancy sample suggesting a hormonal influenced regulation and further a possible protection mechanism to avoid a false implantation of the embryo within the fallopian tube.

With regards to the event of extrauterine tubal pregnancy in human it could be possible that not only the absence of IL-1ra but furthermore an inappropriate ratio of IL-1β and IL-1ra favoring IL-1β may serve as one underlying molecular mechanism.

Based on those findings in human as well as in the animal studies the IL-1β system appears to be involved in the establishment of pregnancy although the exact magnitude is still questionable due to the conflicting data in the literature. Furthermore, rigorous species-specific differences might attribute to an inconsistency in the importance of the IL-1 system in implantation as well. The finding that IL-1R1 is expressed in both cycle phases independent of a pregnancy in addition to the embryos own IL-1β expression supports the concept of an early embryo–maternal dialogue facilitating a proper implantation process.

Declaration of DM Baston-Büst´s role:

Participation in experimental outline, RNA isolation, immunohistochemistry, interpretation of the data, participation in writing of the manuscript

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Interleukin-1 system in the human fallopian tube—No spatial but a temporal regulation of mRNA and protein expression

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Spatial and temporal expression pattern

ABSTRACT

The human fallopian tube provides the environment for the first 5 days of embryonic development *in vivo*. The IL-1 system is involved in human embryo implantation. This study aimed to investigate IL-1β, IL-1ra and IL-1R tI expression within the length of the human fallopian tube on mRNA- and protein-level in samples from proliferative versus secretory phase, postmenopause (PMP) samples and samples from intra- (IUP) and extrauterine pregnancies (EUP) to examine possible spatial and hormonal induced changes (fimbrial, ampullary and isthmic tube segments). On mRNA-level, IL-1β was expressed in all samples except in PMP. IL-1R tI could be detected in all samples whereas IL-1ra was only expressed in secretory phase and the IUP sample. Immunohistochemically we could detect IL-1β and IL-1R t1 protein in all proliferative and secretory phase samples with maximum intensity in secretory phase samples whereas IL-1ra was expressed in secretory phase samples only. Overall no spatial but temporal differences possibly due to hormonal changes could be observed suggesting a precise regulation of the IL-1 system, especially for IL-1ra and moreover a stable molecular architecture within the full length of the fallopian tube.

1. Introduction

The factors involved in the complex embryonic maturation and in the embryo–maternal dialogue resulting in eutopic embryonic implantation are poorly understood. The human fallopian tube is the place of fertilization and also provides the natural environment for the first 5 days of embryonic development (Hess et al., 2007b). It is, as well, the most common place for ectopic implantation. The preimplantation embryo produces several factors during its development to signal its presence to the maternal organism. Appropriate interaction between the preimplantation embryo and the maternal system is at least partly controlled by paracrine cytokines (Hess et al., 2007a; Popovici et al., 2006). Knowledge of expression patterns of these cytokines could possibly help to gain a better insight of the physiological course of implantation. Although multiple studies have examined the existence and influence of various cytokines at the embryonic–maternal interface (Hess et al., 2007a; Popovici et al., 2006), as yet, little is known about cytokine production by the human fallopian tube (Brabec and Hill, 2000).

The Interleukin-1-system is composed of two agonists, Interleukin-1α (IL-1α) and Interleukin-1β (IL-1β), one antagonist, the Interleukin-1 receptor antagonist (IL-1ra) and two membrane-bound receptors, Interleukin-1 receptor type I (IL-1R tI) and II (IL-1R tII) (Dinarello, 1994; Dimitriadis et al., 2005). All components of the IL-1 family in humans are located on chromosome 2 and the DNA-, RNA- and protein-structures are all well characterized for many species. Both agonists are initially synthesized as precursor proteins of 31 kDa. The mature proteins have a molecular weight of 17 kDa and although the amino acid sequences have a similarity of only ~22%, they induce the same biological responses (Dower et al., 1986). There is also a high similarity between the cDNA-sequences of IL-1α and IL-β in mice and human (March et al., 1985). Interleukin-1 receptors type I and II both possess a transmembraneous domain and their extracellular portions are homologous with similar binding affinities for the agonists and antagonist. There is also a soluble form of the IL-1R tII. The IL-1 receptor type I which is found in low numbers on almost all cell surfaces whereas IL-1R tII is found primarily on white blood cells. Only the binding of either IL-1α or IL-β to the IL-1 receptor type I results in signal transduction (Sims et al., 1993) with receptor type II and the soluble IL-1 receptor acting as competitors of the receptor type I (Colotta et al., 1993). The IL-1 receptor antagonist binds with a high affinity to both receptors and prevents signal transduction by IL-1α and IL-β (Hannum et al., 1990).

The IL-1 system is intimately involved in embryonic implantation. In humans, the IL-1R tI has been detected in total human endometrium (Simon et al., 1993b) and, more specifically, in endometrial epithelial cells with a maximum mRNA- and protein-

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expression during the luteal phase (Simon et al., 1993a)—the time of embryonic attachment and implantation. IL-1β-mRNA was detected in secretory human endometrium beginning on day 23 of the menstrual cycle (Kauma et al., 1990). In the last years, all major components of the IL-1-system, namely IL-1β, IL-1ra and IL-1R tI were detected immunohistochemically in single preimplantation embryos (De los Santos et al., 1996). *In vitro* fertilized, cultured human embryos have been shown to produce both IL-1α and IL-1β. High concentrations [>60 pg/ml and >80 pg/ml] of these cytokines in culture media have been correlated with successful implantation after intrauterine transfer of these embryos (Sheth et al., 1991), although other authors could not detect IL-1α or IL-β in culture fluids of human embryos (Seifer et al., 1993). In mice, IL-1α and IL-1β have been detected and localized at mRNA- and protein-level in endometrial endothelial cells (Tackacs et al., 1988) in increasing levels from day 3 of pregnancy peaking between days 4 and 5 (De et al., 1993). Blastocyst implantation is known to occur late on day 4. It was shown that systemically administered recombinant human IL-1ra given intraperitoneally from day 3 to day 6 of pregnancy inhibited embryonic implantation in mice (Simon et al., 1994a). It was also demonstrated that *in vitro* cultured single mouse embryos at various stages of preimplantation development express different patterns of mRNA for IL-1β, IL-1ra and IL-1R tI (Simon et al., 1994b).

In summary, there is an increasing body of evidence that the IL-1 system is involved in early embryonic development and implantation. The aim of our study was to detect a potentially spatial and temporal IL-1β-, IL-1ra- and IL-1R tI-expression pattern on mRNA- and protein-level in human fallopian tubes and to describe possible hormonally regulated changes since these changes might influence the time and place of embryonic implantation.

2. Materials and methods

2.1. Patients

Patients who underwent hysterectomy or tubal surgery for reasons other than cancer (except one patient with intrauterine pregnancy diagnosed of cervical cancer) were asked to participate in this study. Each participating patient signed an informed consent that was prior approved by the human investigations committee of the Heinrich-Heine University Medical School. Menstrual phase was determined by the patient's history and dating was verified by histological examination of the endometrium according to Noyes et al. (1950) and serum-levels of estradiol, progesterone, LH and FSH were measured. The indications for surgery were as follows: hysterectomy for benign reasons: 19 patients; ectopic tubal pregnancies: 3 patients and one patient received a radical hysterectomy in the 9th week of gestation due to cervical cancer. The mean age of the patients was 39 ± 7.9 years, range 26–51 years. Tissue samples from the fimbrial, ampullary and isthmic parts of the fallopian tubes were collected from both sides. Tissue samples were than split in half: one half was fixed in 4% paraformaldehyde and later processed for immunohistochemistry, the other half was snap-frozen in liquid nitrogen and stored at −80 °C until RNA-extraction.

2.2. RNA isolation

The isolation of RNA from the tissue samples was done as described previously (Chomczynski and Sacchi, 1987) with the RNA-STAT-60 reagent (Tel-Test "B" Inc., Friedenswood, TX, USA). Briefly, tissue samples were washed three times in phosphate buffered saline (PBS, GibCo BRL, Grand Island, NY, USA) to remove blood contamination. 100 mg tissue was homogenized in 1 ml of RNA-STAT-60 reagent. Total RNA was separated from DNA and proteins by adding chloroform and precipitated using isopropanol. Precipitate was washed two times in 75% ethanol, air dried and resuspended in diethylpyrocarbonate- (DEPC) treated dH_2O. The amount and purity of the isolated RNA was quantitated by spectrophotometry in a GenQuant RNA/DNA calculator (Pharmacia Biotech Ltd., Cambridge, UK).

2.3. Primers for reverse transcription (RT) and polymerase chain reaction (PCR)

Sequences of cDNA-clones for the mRNAs that should be detected in human fallopian tubes (β-actin, IL-1β, IL-1ra and IL-1R tI) were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH, Internet address: http://www.ncbi.nlm.nih.gov/sites/entrez). One set of primer-sequences was found with the help of the program OLIGO 4.1 Primer Analysis Software (National Bioscience, Plymouth, MN, USA) and synthesized by MWG Biotech (MWG Biotech AG, Ebersberg, Germany). To ensure that the product detected resulted from amplification of cDNA rather than contaminating genomic DNA, primers were designed to cross intron/exon boundaries. The human β-actin primers that were used to amplify an internal standard were obtained from Clontech Laboratories Inc., Palo Alto, CA, USA. The primer-sequences, locations on the cDNA and the sizes of the amplified fragments are listed in Table 1.

2.4. Reverse transcription (RT)

For each mRNA to be detected, 20 μl RT-MasterMix were prepared (5 mM $MgCl_2$, 1× RT Buffer, 1 mM dNTPs (each), 2.5 μM Oligo $d(T)_{16}$, 1 U/μl RNase Inhibitor, 2.5 U/μl MuLV Reverse Transcriptase [all Applied Biosystems, Foster City, CA, USA], and 1 μg total RNA, DEPC-treated H_2O and 20 μl) and filled into a 0.5 ml thin wall PCR-tube (Applied Scientific, South San Francisco, CA, USA). RT-MasterMix in PCR-tubes was covered with 50 μl of light white mineral oil (Sigma, St. Louis, MO, USA) and kept on ice until the RT.

The RT-reaction was carried out in the DNA Thermal Cycler 480 by using a program with the following parameters: 42 °C, 15 min; 99 °C, 5 min; 4 °C, ∞. After the reaction was complete, samples were stored at −20 °C until the PCR.

2.5. Polymerase chain reaction (PCR)

2.5 μl RT-products were mixed with 77.5 μl PCR-Mastermix containing 1.25 mM $MgCl_2$, 1 × PCR-Buffer, 0.125 μM 3′ and 5′ primer-mix for either IL-1β, IL-1ra, IL-1R tI or β-actin, 1 mM dNTPs (each), 1.5 U/μl AmpliTaq® DNA Polymerase, DEPC-treated dH_2O ad 80 μl (Applied Biosystems) and the reaction-mix was covered with 50 μl light white mineral oil, put in the DNA Thermal Cycler 480 and heated to 95 °C for 5 min to activate the enzyme. After completion of 30 cycles of 94 °C for 45 s, 54 °C for 45 s and 72 °C for 60 s, the reaction was terminated at 72 °C for 7 min and cooled down to 4 °C. PCR-products were stored at −20 °C until 2% agarose-gel electrophoresis was carried out in the presence of ethidiumbromide. After completion of electrophoresis, the agarose-gel was analyzed on the GelDoc 1000 system (Bio-Rad Laboratories, Hercules, CA, USA). DNA-size calculation was carried out by using the Molecular Analyst Software (Bio-Rad Laboratories).

2.6. Immunohistochemical staining procedure

The immunostaining procedure was performed on fallopian tubes sections by the avidin–biotin–peroxidase (ABC) method. Serial sections from each sample were mounted on slides, fixed in formalin and embedded in paraffin. The first section was processed for hematoxylin–eosin staining.

Sections were deparaffinized in xylene, rehydrated through graded alcohols, and washed in phosphate-buffered saline (PBS; Sigma). Endogenous peroxidases were blocked with 1% H_2O_2 in 96% methanol, and non-specific binding was blocked with non-fat milk 5% in PBS at room temperature. After washing with 0.05% Tween®20 in PBS (PBS-T; Sigma), the slides were incubated at room temperature for 30 min with the primary antibodies: monoclonal mouse anti-human IL-1β antibody, monoclonal mouse anti-human IL-1R tI antibody, and polyclonal rabbit anti-human IL-1ra (all from Genzyme Corp., Cambridge, MA, USA) at 20 μg/ml, 50 μg/ml, and

Table 1
Primers for synthesis of cDNA clones of human β-actin, IL-1β, IL-1ra and IL-1R tI.

mRNA	Size of amplified fragment [bp]	Position of primers on cDNA	3′/5′-end	Sequence of oligonucleotide (5′ → 3′)
β-Actin	838	294–325	5′-end	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG
		1100–1131	3′-end	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC
IL-1β	549	101–122	5′-end	CAG TGA AAT GAT GGC TTA TTA G
		628–648	3′-end	CTT TCA ACA CGC AGG ACA GGT
IL-1ra	424	91–114	5′-end	CAG AAG ACC TCC TGT CCT ATG AGG
		498–514	3′-end	GCTT GTG CAG AGG AAC CA
IL-1R tI	284	341–361	5′-end	AAG GTG GAG GAT TCA GGA CAT
		604–624	3′-end	AGC CTA TCT TTG ACT CCA CTA

20 μg/ml, respectively. Control incubations included deletion of the primary antibody. After rinsing with PBS-T, sections were incubated with a secondary antibody at room-temperature for 30 min: anti-rabbit IgG (whole molecule) biotin conjugate (200 μg/ml; Sigma) and anti-mouse IgG biotin conjugate (Vector Laboratories INC, Burlingame, CA, USA) at 150 μg/ml. Histological sections were incubated with Vectastain ABC Kit (Vector Laboratories) reagents for 60 min at room temperature. Immunoreaction products were visualized by incubating sections with the substrate solution in 0.1 M Tris–HCl pH 8.2 buffer blocking alkaline phosphatase activity with levamisole. Slides were counterstained with 25% of hematoxylin, then cleared, cover slips applied and finally examined by light microscopy. Positive staining by the primary antibody was indicated by a pink precipitate. The relative intensity of the immunostaining was evaluated by two of the authors in a double-blind manner as absent (0), weakly positive (+), moderate (++) or intense (+++) for biopsy specimens from each portion and each phase of the menstrual cycle.

3. Results

3.1. RT-PCR of spatial expression pattern throughout the menstrual cycle

All 19 collected oviduct samples from the proliferative ($n = 8$) and secretory ($n = 9$) phase of the cycle and postmenopausal ($n = 2$) women were divided into fimbrial, ampullary and isthmic parts and investigated separately by RT-PCR. From the total of 19 samples all were positive for β-actin mRNA expression and therefore considered for further investigation. The following investigation of IL-1β, IL-1ra and IL-1R tI expression showed that the presence or absence of the respective gene was constant regarding the spatial distribution. This shows that the gene expression of IL-1β, IL-1ra and IL-1R tI within the length of the fallopian tube has no spatial difference (Fig. 1).

3.2. RT-PCR of temporal expression pattern throughout the menstrual cycle

All 23 collected oviduct samples from the proliferative ($n = 8$) and secretory ($n = 9$) phase of the cycle and from postmenopausal ($n = 2$) women and extra- ($n = 3$) and intrauterine ($n = 1$) pregnancies were separately investigated by RT-PCR for temporal expression patterns of IL-1β, IL-1ra and IL-1R tI. From the total of 23 samples, all were positive for β-actin mRNA expression and therefore considered for further investigation. The following investigation of IL-1β, IL-1ra and IL-1R tI expression showed that there was a temporal and therefore most likely hormonal regulation of two of the target mRNAs. IL-1R tI was expressed in all samples regardless the hormonal situation. IL-1ra mRNA could be detected in samples of the secretory phase and in intrauterine pregnancy, whereas IL-1β could be detected in proliferative and secretory phase fallopian tubes and in samples of intra- and extra-uterine pregnancy. This shows that the gene expression of IL-1β, IL-1ra and IL-1R tI is temporally regulated within the human cycle (Figs. 1 and 2). Additionally IL-1β is expressed not only in all different hormonal situations investigated but also in oviducts from intra- and extrauterine pregnancies.

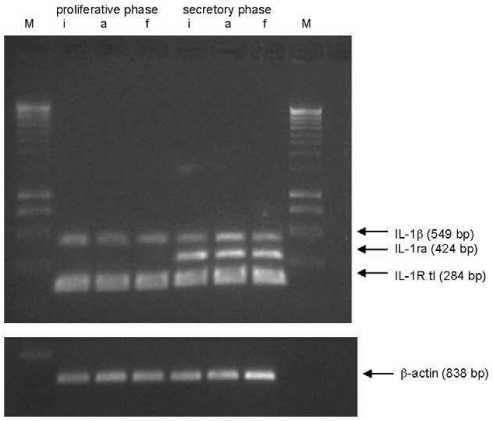

Fig. 1. Representative RT-PCR-gel electrophoresis for IL-1β (549 bp), IL-1ra (424 bp) and IL-1R t1 (284 bp) for three samples each showing that the gene expression of IL-1β, IL-1ra and IL-1R t1 within the length of the fallopian tube has no spatial difference (β-actin with 838 bp serves as control). All oviduct samples were divided into fimbrial, ampullary and isthmic parts and investigated separately by RT-PCR. From the total of 19 samples, all were positive for β-actin mRNA expression (M, DNA size marker; p, proliferative; s, secretory; f, fimbrial; a, ampullary; i, isthmic).

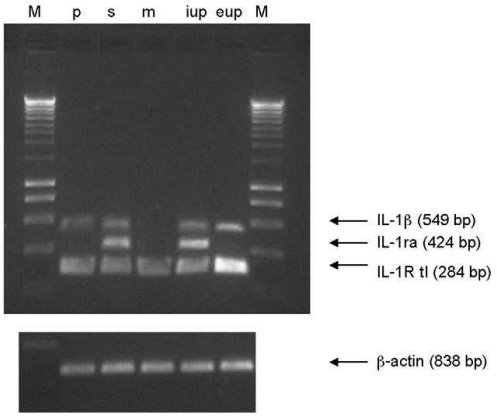

Fig. 2. Representative RT-PCR gel electrophoresis for temporal expression patterns of IL-1β, IL-1ra and IL-1R tI showing a temporal and therefore most likely hormonal regulation of IL-1β and IL-1ra, whereas the receptor was expressed in all samples. From the total of 23 samples, all were positive for β-actin mRNA expression (M, DNA size marker; p, proliferative; s, secretory; m, menopausal; iup, intrauterine pregnancy; eup, extrauterine pregnancy).

3.3. Immunohistochemistry

The protein expression of IL-1β, IL-1ra and IL-1R tI of proliferative phase versus secretory phase samples was evaluated using immunohistochemistry. Fig. 3A–H shows representative samples from fallopian tubes of the secretory phase whereas Fig. 3I–P shows representative samples from proliferative phase fallopian tubes. Panel A–G and I–O are magnified 200×, panel B–H and J–P 400×. Panels A and B represent the control of secretory phase oviducts, panel I and J represent the negative control (omission of first antibody) of proliferative phase oviducts. We could show a strong expression of IL-1β foremost in the luminal epithelium and also in the small vessels endothelium of secretory phase oviducts (Fig. 3C and D), whereas the staining for IL-1β in the luminal epithelium of proliferative phase oviducts was less intense and only a weak staining was localized in the small vessels endothelium (Fig. 3K and L). The IL-1R tI was shown to be expressed in the luminal epithelium, small vessels endothelium and glandular epithelium of secretory phase samples (Fig. 3E and F). In comparison the proliferative sample showed only a sporadic staining for the IL-1R tI in the luminal epithelium and no staining either in the vessel endothelium or in the glandular epithelium (Fig. 3M and N). Fig. 3G and H show an intense staining of IL-1ra in the luminal epithelium and the small vessel endothelium of secretory phase oviducts whereas a staining for IL-1ra in proliferative oviducts was completely absent revealing a strict temporal expression of IL-1ra.

No staining was observed in the absence of either primary or secondary antibodies.

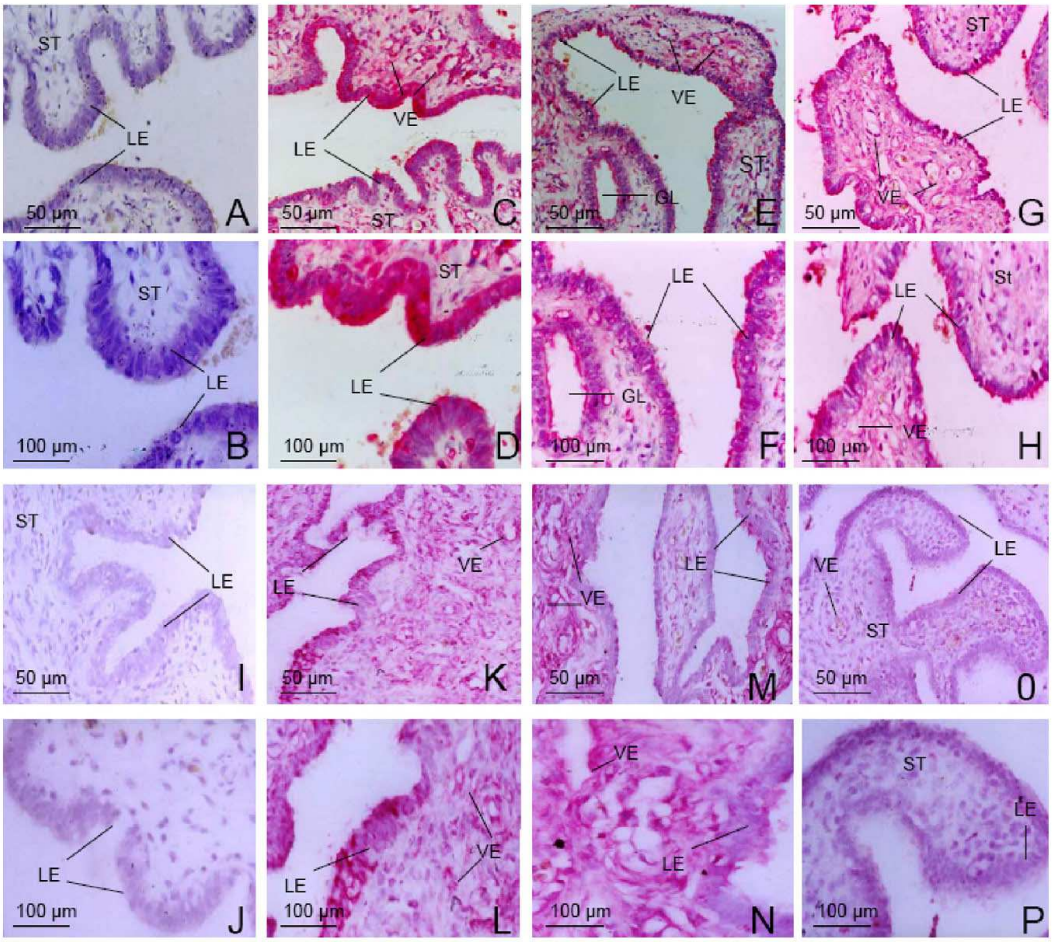

Fig. 3. Immunohistochemistry staining of representative samples from fallopian tubes of the secretory (A–H) and proliferative phase (I–P) (panel A–G and I–O 200× magnification, panel B–H and J–P 400× magnification). Panels A and B represent the negative control of secretory phase oviducts, panel I and J represent the negative control (no first antibody) of proliferative phase oviducts. Note that IL-1β is strong expressed in the luminal epithelium and also in the small vessel endothelium of secretory phase oviducts (C and D), whereas the staining for IL-1β in the luminal epithelium of proliferative phase oviducts was less intense and only a weak staining was localized in the small vessel endothelium (3 K and L). IL-1R t1 is expressed in the luminal epithelium, small vessel endothelium and glandular epithelium of secretory phase samples (E and F). In comparison, the proliferative sample showed only a sporadic staining for the IL-1R t1 in the luminal epithelium and no staining either in the vessel endothelium or in the glandular epithelium (M and N). G and H show an intense staining of IL-1ra in the luminal epithelium and the small vessel endothelium of secretory phase oviducts whereas a staining for IL-1ra in proliferative oviducts was completely absent.

These protein data support the previous mRNA data showing an expression of IL-1β in fallopian tubes of both cycle phases with an increase in the secretory phase as well as a pronounced expression of IL-1R tl in secretory phase oviducts. However, IL-1ra expression was seen in secretory phase samples only.

4. Discussion

Factors of the embryo–maternal communication that have an influence on maturation and transport of an embryo as it transverses the oviducts are crucial for a successful implantation (Hess et al., 2007a). The fallopian tube on the maternal side plays an important role in providing the place for fertilization as well as an active component for transport and early development of the embryo. Within the oviduct, remarkable changes occur in dependence to the sex steroids estradiol in the proliferative phase and progesterone in the post-ovulatory secretory phase indicating a hormonal cyclicity of the oviduct comparable to the endometrium (Gardner et al., 1996). A growing body of evidence suggests that chemokines of the IL-1 family play an important role at the feto-maternal interface (Krussel et al., 2003). Therefore, the knowledge of normal expression patterns of the IL-1 family members could help in understanding the physiological processes leading to proper maturation and transport of the blastocyst within the fallopian tube and subsequently to a successful intrauterine implantation. Since we have shown before that the human embryo produces IL-1β when cultured *in vitro* (Krussel et al., 1998), it is reasonable that it also produces IL-1β during its passage through the fallopian tube. Consequently, it is very likely that the fallopian tube as the early communication partner for the developing embryo itself expresses components of the IL-1 family. Therefore, our objective was to first investigate the spatial expression pattern of IL-1β, IL-1ra and IL-1R

tI in the human fallopian tube and second to elucidate a possible hormonal influence. Furthermore we wanted to explore whether we could find a hint for an abnormal expression pattern or an imbalance of gene expression contributing to a risk of an extrauterine tubal pregnancy.

All collected oviduct samples from the proliferative and secretory phase of the cycle as well as the postmenopausal samples were divided into fimbrial, ampullary and isthmic parts before investigation and prepared separately. This led us to the observation that regardless of the original spatial belonging of the oviduct part, the mRNA expression of IL-1β, IL-1ra and IL-1R tI showed no differences at all. These findings on the mRNA-level were also proven on the protein-level by immunohistochemistry (data not shown). The data support the notion that although the oviduct is compromised of a changing architecture within its full length, the molecular base of gene expression remains the same in all parts of the fallopian tube. Therefore, possible differences in gene expression regarding to the biopsy technique are erased which makes studies involving different parts of the oviduct comparable.

Furthermore, our aim was to elucidate whether the sex steroids estrogen and progesterone have an influence on the temporal expression of IL-1β, IL-1ra and IL-1R tI during the reproductive phase. Therefore, fallopian tubes from the proliferative and secretory phase of the menstrual cycle as well as oviducts from postmenopausal women were investigated. This showed that IL-1β was only present in samples from the proliferative and secretory phase but in none of the postmenopausal oviducts suggesting a regulation by sex steroids and a role in the process of human reproduction. These data highlight that the oviduct and the uterus underlie partially common most likely hormonally driven regulatory expression phenotypes since a study has shown that IL-1β is expressed as well by epithelial and stromal endometrium cells throughout the cycle (Simon et al., 1993a). In unison with our immunohistochemistry results they detected an increase of IL-1β immunostaining from proliferative to secretory endometrium suggesting an additional role in supporting the implantation within the endometrium during the window of implantation. On the contrary, another study primarily focused on the expression of IL-1α by endometrial stromal cells reports a hormonally controlled expression of IL-1α but not for IL-1β or IL-1ra (Pretto et al., 2008). Furthermore, it was shown that incubation of endometrial stromal cells with IL-1β upregulates the expression of IL-1β and IL-1ra (Huang et al., 2001) suggesting that besides a hormonal regulation chemokines seem to influence the expression of IL-1 family members in endometrial stromal cells which could be likewise for the tube. In addition an immunohistochemical approach revealed IL-1β staining in the villous cytotrophoblast, syncytiotrophoblast and maternal decidual cells, suggesting an autocrine/paracrine role during the human implantation (Simon et al., 1994b). In contrast to the ligand, we detected mRNA expression of its receptor IL-1R tI in all samples investigated. The immunostaining showed a less intense staining in proliferative phase samples compared to secretory phase oviducts suggesting at least a moderate influence of the sex hormones. Furthermore it provides new evidence that the preimplantation embryo is able to communicate with the maternal side already during its transit through the fallopian tube by its own IL-1β secretion in consent to a study by Strakova et al. (2005) simulating endometrial changes of an early pregnancy in baboons by *in vivo* infusion of hCG and IL-1β.

In contrast, IL-1ra mRNA was only detected in progesterone dominated secretory phase samples and in the intrauterine pregnancy sample suggesting a hormonal influenced regulation and further a possible protection mechanism to avoid a false implantation of the embryo within the fallopian tube. The IL-1ra expression seems to be a rather systemic effect independent of the embryos presence since it was equally expressed in both sides of the fallopian tubes of each patient in the secretory phase. IL-1ra's possible protective effect becomes even more likely in the context that IL-1ra was not only present in all secretory phase samples and the one investigated sample with an intrauterine pregnancy but did not occur in anyone of the three tested fallopian tubes with an extrauterine pregnancy. This hypothesis is furthermore supported by findings of other groups describing a decrease in IL-1ra expression in the human endometrium at the time of implantation to possibly facilitate the embryos invasion into the maternal endometrium (Simon et al., 1995). Another study showed increased levels of IL-1ra in endometrial stromal cells of the secretory phase (Fukuda et al., 1995). Those differences though might be explained by the fact that the first study investigated the mRNA and protein expression for secreted IL-1ra and intracellular IL-1ra whereas the second study did not differentiate between those two forms. The findings of the first study positively correlate with a study in mice where systemically administered IL-1ra prevented embryonic implantation (Simon et al., 1994a). However, this distinct inhibition of implantation might have been a mouse strain specific effect as a study by Abbondanzo et al. (1996) disproved the latter finding in IL-1R tI female knockout mice showing no deficiency in fertility besides a slightly smaller litter size as well as Cohen and Pollard (1998) could not show noticeable fertility abnormalities in male IL-1R tI knockout mice. On the other hand a recent knock out study with male$^{IL\text{-}1ra-/-}$ mice showed a reduced capacity to fertilize oozytes of wild type females *in vitro* and a reduced litter size when mated with wild type females (Ganaiem et al., 2009). The discrepancy of those studies might have been evolved due to the fact that Abbondanzo et al. e.g. have used an outbread strain with a mutated gene which are known to possibly have an influenced phenotype. Otherwise it might be conceivable that subsequent events in the signaling cascade after blocking a receptor with the receptor antagonist compared to a knock out of the receptor itself may vary and possibly lead to inconsistent results as shown above when data of IL-1R tI knock out studies are compared with IL-1ra knock out studies. With regards to the event of extrauterine tubal pregnancy in human it could be possible that not only the absence of IL-1ra but furthermore an inappropriate ratio of IL-1β and IL-1ra favoring IL-1β may serve as one underlying molecular mechanism.

So far most investigations regarding the molecular base of extrauterine pregnancies were conducted in the mouse or baboon model. Therefore little is known in the human system. Huang et al. (2005) investigated fallopian tubes from extrauterine pregnancies versus normal healthy control tubes from tubal ligations. The PCR approach showed partially varying results from our data with an upregulation of IL-1R tI and IL-1ra mRNA simultaneous to an IL-1β downregulation in tubes with ectopic pregnancies. The study by Huang et al. applied a quantitative PCR approach using a competitor compared to the qualitative method which was used in this study showing no upregulation of IL-1RtI and a downregulation of IL-1β but rather the presence of both in fallopian tubes of extrauterine pregnancies. Presumptions about a regulation cannot be drawn therefore. However the upregulation of IL-1ra in extrauterine tubes is opposed to the absence of IL-1ra in extrauterine and healthy proliferative phase tubes as occurred in the present study. Both studies have investigated a relatively small number of fallopian tube samples most likely due to low availability which might explain the partly varying results.

Based on those findings in human as well as in the animal studies the IL-1β system appears to be involved in the establishment of pregnancy although the exact magnitude is still questionable due to the conflicting data in the literature. Furthermore, rigorous species-specific differences might attribute to an inconsistency in the importance of the IL-1 system in implantation as well.

To our knowledge this is the first study to report that the gene expression profile is not altered within the different anatomical

parts of the fallopian tube showing a constant gene expression pattern of all IL-1β system factors throughout the complete length of the oviduct investigated. Additionally, the restricted IL-1ra expression in secretory phase fallopian tubes and absence in extrauterine pregnancy tube samples might be an important mechanism on the molecular base to prevent ectopic pregnancies within the oviduct which needs further investigation. The finding that IL-1R tI is expressed in both cycle phases independent of a pregnancy in addition to the embryos own IL-1β expression supports the concept of an early embryo–maternal dialogue facilitating a proper implantation process.

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2.2 Cathepsin S system at the feto-maternal interface (Reproduction 139 (2010), 741-748)

DM Baston-Buest, A Schanz, S Buest, JC Fischer, JS Kruessel, AP Hess

The embryo–maternal dialogue is characterized by the secretion of chemokines, growth factors, matrixdegenerating enzymes, and their inhibitors from the receptive maternal endometrium and the embryo. Recent in vitro culture studies of primary human endometrial cells combined with an imitation strategy for implantation by using trophoblast-conditioned medium and first-trimester trophoblast explants followed by microarray mRNA analyses yielded new insights into the underlying molecular processes of human implantation. One of the statistically significant regulated molecules involved was cathepsin S (CTSS), a cysteine proteinase. In general, cathepsins need to be activated in the acid environment of lysosomes and are sensitively regulated as their release in the extracellular space can be destructive for cells. CTSS is stable and active at neutral and acid pH cathepsins have been reported to activate molecules, e.g. matrix metalloproteinases (MMPs) and E-cadherin, when expressed on the cell surface and therefore participate in angiogenesis and tumor invasion – processes similar to those occurring during embryo implantation. CTSS was found to be activated by interleukin-6 (IL-6) in dendritic cells, which is secreted as one major chemoattractant chemokine in trophoblast cells. A possible source for CTSS in early human pregnancy might be the influx of immunocompetent cells starting in the secretory phase of the menstrual cycle, which also express the vascular endothelial growth factor (VEGF) as one of the most potent angiogenic factors in the first trimester of pregnancy. In order to avoid cellular damage and uncontrolled digestion, the activity of cathepsins needs to be regulated carefully, which is controlled by the cystatin (CST) superfamily.

This superfamily of evolutionarily conserved proteins, which are involved in the inhibition and regulation of cysteine proteinases, neutrophil chemotaxis, tissue inflammation, hormone processing, reflecting essential mechanisms of embryo implantation, can be divided into three subcategories: cytosolic type I cystatins, especially stefins A and B; secreted type II

cystatins found in many body fluids, such as CST3 (CstC), CST5 (CST10, CstD), and CST7 (CstF); and kininogens as type III cystatins, which are localized in plasma.

Within the human secretory phase and early embryonic adhesion phase, the invasion of different immune cells is known to be important for a successful implantation. Recently, a shift in the allocation of human dendritic cells during the normal cycle was reported. CST7 is limited in its expression to cells of the immune system, e.g. natural killer (NK) cells and dendritic cells, and therefore potentially involved in the process of embryo implantation.

Our present study is the first to investigate a possible cycle-dependent regulation of CTSS and its inhibitors *in vivo* in the female mouse uterus and in early pregnancy, blastocysts, and implanted embryos in order to determine the role of this proteinase and inhibitor interaction in the early processes of pregnancy establishment. In addition, we determined the ratio of immunocompetent cells as a source for CTSS and CST3 and CST7 in nonpregnant uteri and decidua on day 9 of pregnancy since murine blastocysts express the mRNA for the chemoattractive IL-6.

From a total of 156 blastocysts examined, 143 were positive for b-actin mRNA expression and therefore considered for further examination. We detected mRNA for CTSS in only two early blastocysts potentially resulting from a maternal carry over, because no other developmental stage and even not the pools showed a Ctss mRNA expression at all. The cysteine proteinase inhibitors Cst7 and Cst3 were expressed in 74 and 143 blastocysts. However, the mRNA for IL-6 could be detected in 95 of all blastocysts examined.

The expression of CTSS and CST3 protein was determined in murine uteri in estrogen- and progesterone-dominated phase, in day 3 blastocyst *in utero* and in pregnancies with implantation sites using immunohistochemistry. Both the protease CTSS and its inhibitor CST3 were localized in the endothelia of glands and vessels in estrogen- and progesterone-dominated murine uteri with a higher degree in the progesterone-dominated uteri. The expression of CTSS increased in the endometrium of pregnant mice, but was very little in blastocysts and was predominant in the pregnant endometrium next to the decidua. On the

contrary, the expression of CST3 decreased from the estrogen to progesterone dominated phase, but there was an intense staining of the blastocysts on day 3 and the endometrium and the decidua directly adjacent to the embryo on day 7 pc. The inhibitor CST7 occurred with the same staining intensity in both phases of the cycle. As was shown for CST3, an intense staining for CST7 occurred in the blastocysts and at the sight of the embryo and adjacent decidua as well as in the glandular epithelium within most parts of the remaining decidua.

In order to specify the localization of the proteins of interest (POIs) seen in immunohistochemistry, flow cytometry versus immunocompetent cells was performed. CD3 served as a marker for mature T- and NK T-cells in mice, CD4 characterizes MHC-II T-cells and subpopulations of NK T-cells, CD8 is expressed on macrophages, MHC-I T-cells, which belong to the cytotoxic T-cells, and dendritic cells. Uterine NK (uNK) cells are described as CD3-negative ($CD3^-$) and CD122-positive ($CD122^+$).

There was an obvious influx of $CD3^-/CD122^+$ cells accumulating CTSS and CST7. $CD4^+$ cells remained nearly in an identical number – including effector T (Th) cells – with a complete loss of CST7-expressing $CD4^+$ cells in decidua. Furthermore, there was a decline in $CD8^+$ cells, which include cytotoxic T cells (Tc) and dendritic cells. All detected $CD8^+$ cells in decidua lacked CTSS expression, but showed an increased expression of both inhibitors. Additionally, the number of gated cells for $CD3^+$ decreased but the expression level for POIs remained similarly with almost 90%.

The highly coordinated interactive process between the receptive maternal endometrium and the invading embryo via early feto-maternal communication through an array of chemokines, growth factors, and proteinases seems to be essential for the establishment of pregnancy.

Declaration of DM Baston-Büst´s role:

Experimental outline, isolation of blastocyst, reverse transcription and PCR, immunohistochemistry, FACS analysis, interpretation of the data, writing of the manuscript

RESEARCH

The embryo's cystatin C and F expression functions as a protective mechanism against the maternal proteinase cathepsin S in mice

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Abstract

A successful implantation of a mammalian embryo into the maternal endometrium depends on a highly synchronized fetal–maternal dialogue involving chemokines, growth factors, and matrix-modifying enzymes. A growing body of evidence suggests an important role for proteinases playing a role in matrix degeneration and enhancing the embryo's invasive capacity and influencing the mother's immunological status in favor of the conceptus. This study focused on the expression of cathepsin S (CTSS) and its inhibitors in the murine fetal–maternal interface as well as the detection of the cellular sources of either proteinase and inhibitors. Nested RT-PCR for detection of embryonic mRNAs, immunohistochemistry of maternal and fetal tissues in B6C3F1 mice, and FACS analysis for determination of immunocompetent cell population were applied. This study shows that the cysteine proteinase CTSS is upregulated in the stroma of the implantation site, and that pregnancy induces an influx of CTSS-positive uterine natural killer cells. Compared to maternal tissues, the CTSS inhibitors cystatin F and C, but not the proteinase itself, are expressed in blastocysts. In conclusion, CTSS underlies a hormonal regulation in the maternal tissue and therewith most likely supports the embryonic implantation. The invading embryo regulates the depth of its own invasion through the expression of the cathepsin inhibitors and furthermore, interleukin-6 to activate CTSS in maternal tissues. Additionally, the observed decrease in $CD3^+$ cells leads to the hypothesis that cells of the cytotoxic T-cell group are down-regulated in the decidua to support the implantation and ensure the survival of the embryo.

Reproduction (2010) **139** 741–748

Introduction

The embryo–maternal dialogue is characterized by the secretion of chemokines, growth factors, matrix-degenerating enzymes, and their inhibitors from the receptive maternal endometrium and the embryo (Paria *et al.* 2001). The early fetal–maternal communication in mammals seems to be a very crucial symphony of a vast variety of molecules. Recent *in vitro* culture studies of primary human endometrial cells combined with an imitation strategy for implantation by using trophoblast-conditioned medium and first-trimester trophoblast explants followed by microarray mRNA analyses yielded new insights into the underlying molecular processes of human implantation (Popovici *et al.* 2006, Hess *et al.* 2007).

One of the statistically significant regulated molecules involved was cathepsin S (CTSS), a cysteine proteinase. In general, cathepsins need to be activated in the acid environment of lysosomes and are sensitively regulated as their release in the extracellular space can be destructive for cells (Halfon *et al.* 1998). However, CTSS is stable and active at neutral and acid pH (Kirschke *et al.* 1989). Cathepsins may be involved in the antigenic processing of proteins and in the arrangement of the invariant chain of major histocompatibility class (MHC)-II molecules (Riese *et al.* 1996). Moreover, cathepsins have been reported to activate molecules, e.g. matrix metalloproteinases (MMPs) and E-cadherin, when expressed on the cell surface and therefore participate in angiogenesis and tumor invasion – processes similar to those occurring during embryo implantation (Gocheva *et al.* 2006, Mohamed & Sloane 2006, Gocheva & Joyce 2007). The modulation of the maternal immune system to accept the embryo as a semi-allograft and the shift in the T-cell response are well-characterized processes in the early establishment of pregnancy. CTSS was found to be activated by interleukin-6 (IL6) in dendritic cells, which is secreted as one major chemoattractant chemokine in trophoblast cells (Kitamura *et al.* 2005). A possible source for CTSS in early human pregnancy might be the

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influx of immunocompetent cells starting in the secretory phase of the menstrual cycle, which also express the vascular endothelial growth factor (VEGF) as one of the most potent angiogenic factors in the first trimester of pregnancy (Reddy *et al.* 1995, Clark *et al.* 1998).

In order to avoid cellular damage and uncontrolled digestion, the activity of cathepsins needs to be regulated carefully, which is controlled by the cystatin (CST) superfamily. This superfamily of evolutionarily conserved proteins, which are involved in the inhibition and regulation of cysteine proteinases, neutrophil chemotaxis, tissue inflammation, hormone processing, reflecting essential mechanisms of embryo implantation, can be divided into three subcategories: cytosolic type I cystatins, especially stefins A and B; secreted type II cystatins found in many body fluids, such as CST3 (CstC), CST5 (CST10, CstD), and CST7 (CstF); and kininogens as type III cystatins, which are localized in plasma (Brown & Dziegielewska 1997, Vray *et al.* 2002). Within the human secretory phase and early embryonic adhesion phase, the invasion of different immune cells is known to be important for a successful implantation

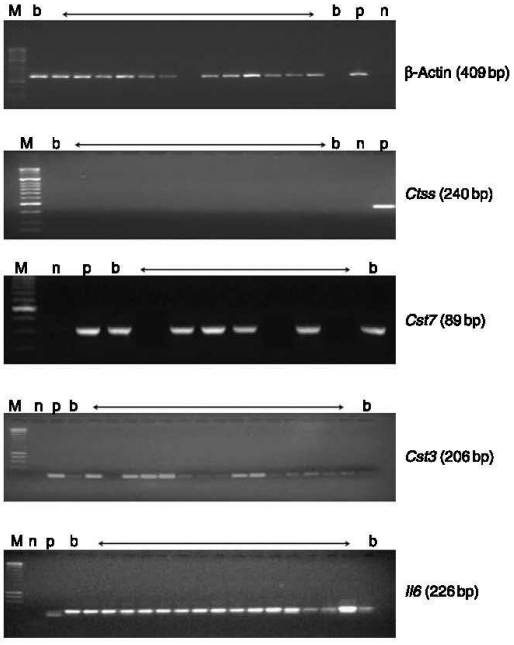

Figure 1 Representative electrophoresis gels of nested RT-PCR analysis of blastocysts reflecting the ratio of expression of each mRNA of interest for β-actin (409 bp) as housekeeping gene and the mRNAs of interest *Ctss* (240 bp), *Cst7* (89 bp), *Cst3* (206 bp), and *Il6* (226 bp). Negative control (n; H_2O), standard bp ladder (M), and positive control (p; mouse ovary cDNA).

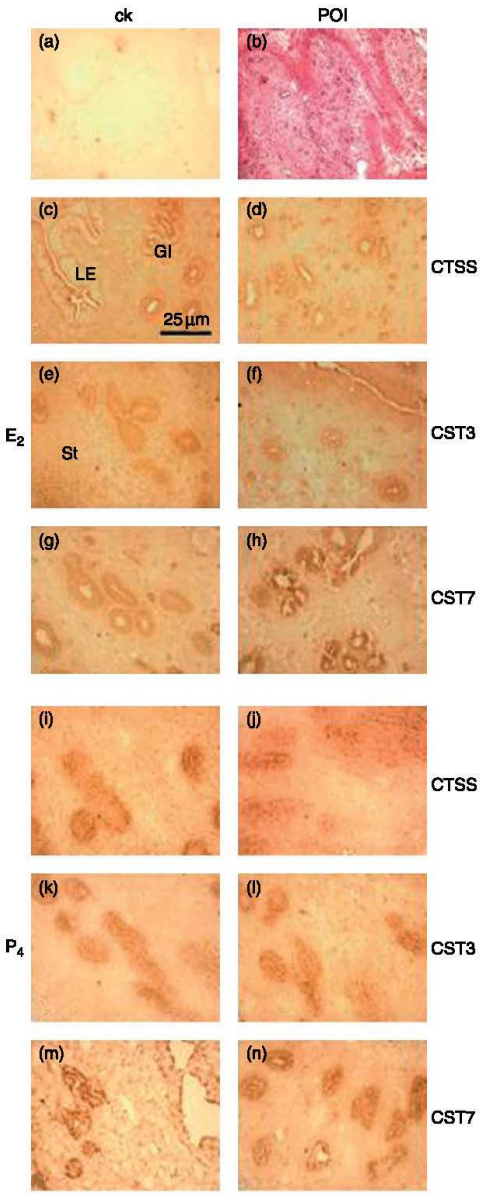

Figure 2 Immunohistochemical staining of CTSS (d and j), CST3 (f and l), and CST7 (h and n) during the estrogen (E_2)- and progesterone (P_4)-dominated phase in female mice (magnification ×400). Cytokeratin was used as an epithelial marker and reference staining (c, e, g, i, k and m). The sections are shown adjacent to each other. Negative staining with nonspecific IgG and hematoxylin–eosin staining are shown above (a and b). ck, cytokeratin; POI, protein of interest; LE, luminal epithelial; GI, gland; St, stroma.

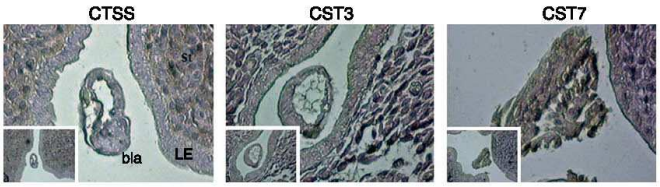

Figure 3 Immunohistochemical staining of CTSS, CST3, and CST7 of murine blastocysts *in utero*. Magnification ×100 (small inserts) and ×450. Cytokeratin was used as an epithelial marker. A negative staining as a control was performed (data not shown). bla, blastocyst; Le, luminal epithelium; St, stroma.

(Lobo *et al.* 2004). Recently, a shift in the allocation of human dendritic cells during the normal cycle was reported (Schulke *et al.* 2008). CST7 is limited in its expression to cells of the immune system, e.g. natural killer (NK) cells and dendritic cells, and therefore potentially involved in the process of embryo implantation (Halfon *et al.* 1998, Nathanson *et al.* 2002).

Cystatin C (CST3) is a common inhibitor of cysteine proteinases mainly in body fluids and was even reported to activate growth (Sun 1989).

Previous studies concerning cathepsins and reproductive processes, e.g. decidualization and blastocyst hatching, focused on the expression of cathepsins B, L, and H and CST3, which are expressed in high concentrations in most tissues (Afonso *et al.* 1997, Jokimaa *et al.* 2001, Sireesha *et al.* 2008).

The results of the microarray studies focusing on modifications at the fetal–maternal interface *in vitro* suggested an involvement of CTSS in embryo implantation (Popovici *et al.* 2006, Hess *et al.* 2007). In view of those data, our present study is the first to investigate a possible cycle-dependent regulation of CTSS and its inhibitors *in vivo* in the female mouse uterus and in early pregnancy, blastocysts, and implanted embryos in order to determine the role of this proteinase and inhibitor interaction in the early processes of pregnancy establishment. In addition, we determined the ratio of immunocompetent cells as a source for CTSS and CST3 and CST7 in nonpregnant uteri and decidua on day 9 of pregnancy since murine blastocysts express the mRNA for the chemoattractive IL6.

Results

RT-PCR and nested RT-PCR

From a total of 156 blastocysts examined, 143 were positive for β-actin mRNA expression and therefore considered for further examination. We detected mRNA for CTSS in only two early blastocysts potentially resulting from a maternal carry over, because no other developmental stage and even not the pools showed a *Ctss* mRNA expression at all (Fig. 1). The cysteine proteinase inhibitors *Cst7* and *Cst3* were expressed in 74 and 143 blastocysts (Fig. 1). However, the mRNA for IL6 could be detected in 95 of all blastocysts examined (Fig. 1).

Immunohistochemistry

The expression of CTSS and CST3 protein was determined in murine uteri in estrogen- and progesterone-dominated phase (Fig. 2), in day 3 blastocysts *in utero* (Fig. 3) and in pregnancies with implantation sites using immunohistochemistry (Fig. 4). Both the protease CTSS and its inhibitor CST3 were localized in the endothelia of glands and vessels in estrogen- and progesterone-dominated murine uteri (Fig. 2) with a higher degree in the progesterone-dominated uteri (Table 1). The expression of CTSS increased in the endometrium of pregnant mice (Table 1 and Fig. 2j), but was very little in blastocysts (Fig. 3a) and was predominant in the pregnant endometrium next to the decidua (Fig. 4a and b). On the contrary, the expression

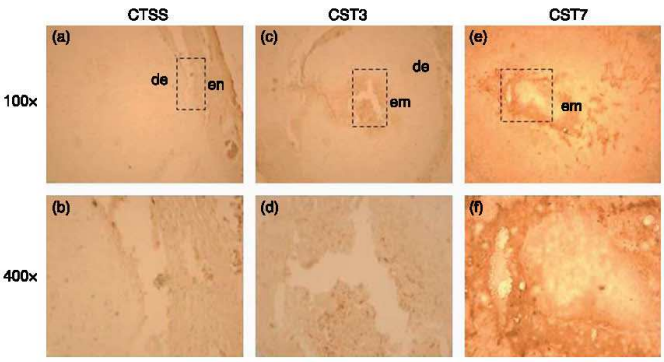

Figure 4 Immunohistochemical staining of CTSS, CST3, and CST7 of day 7 murine implantation sites and decidual–endometrial interface respectively. Magnification ×100 and ×400 as indicated. The squares indicate the parts shown in 400× magnification. de, decidua; em, embryo; en, endometrium.

Table 1 Rating of immunohistochemical staining of murine uteri (stroma, vessels, and glandular epithelium) and 7 days old embryos, absent (−), weakly positive (+), moderate (++), intense (+++) staining.

	CTSS	CST3	CST7	Tissue
E_2-dominated phase	+	++	+	Stroma
P_4-dominated phase	+	+	+	
Implantation site endometrium/myometrium	+	+	+	
E_2-dominated phase	+	+	+	Vessels
P_4-dominated phase	+	+	−	
Implantation site endometrium/myometrium	+	+	+	
E_2-dominated phase	++	++	++	Glandular epithelium
P_4-dominated phase	+++	+	++	
Implantation site endometrium/myometrium	++	++	+	
	−	++		Embryonic site

of CST3 decreased from the estrogen to progesterone dominated phase (Table 1 and Fig. 2l), but there was an intense staining of the blastocysts on day 3 (Fig. 3b) and the endometrium and the decidua directly adjacent to the embryo on day 7 pc (Fig. 4c and d). The inhibitor CST7 occurred with the same staining intensity in both phases of the cycle (Table 1, Fig. 2h and n). As was shown for CST3, an intense staining for CST7 occurred in the blastocysts (Fig. 3c) and at the sight of the embryo and adjacent decidua as well as in the glandular epithelium within most parts of the remaining decidua (Fig. 4e and f).

Flow cytometry

In order to specify the localization of the proteins of interest (POIs) seen in immunohistochemistry, flow cytometry versus immunocompetent cells was performed. CD3 served as a marker for mature T- and NK T-cells in mice, CD4 characterizes MHC-II T-cells and subpopulations of NK T-cells, CD8 is expressed on macrophages, MHC-I T-cells, which belong to the cytotoxic T-cells, and dendritic cells. Uterine NK (uNK) cells are described as CD3-negative ($CD3^-$) and CD122-positive ($CD122^+$; Yadi *et al.* 2008).

First, there was an obvious influx of $CD3^-/CD122^+$ cells accumulating CTSS and CST7 (Table 2). $CD4^+$ cells remained nearly in an identical number – including effector T (T_h) cells – with a complete loss of CST7-expressing $CD4^+$ cells in decidua (Table 2). Furthermore, there was a decline in $CD8^+$ cells, which include cytotoxic T cells (T_c) and dendritic cells. All detected $CD8^+$ cells in decidua lacked CTSS expression, but showed an increased expression of both inhibitors. Additionally, the number of gated cells for $CD3^+$ decreased but the expression level for POIs remained similarly with almost 90% (all Table 2).

Discussion

The highly coordinated interactive process between the receptive maternal endometrium and the invading embryo via early feto-maternal communication through an array of chemokines, growth factors, and proteinases seems to be essential for the establishment of pregnancy. Herein, we focus on the role of the cysteine proteinase CTSS, which was found to be upregulated in decidualized endometrial stromal cells after contact with trophoblast-conditioned medium, and its inhibitors CST7 and CST3 (Hess *et al.* 2007).

CTSS accomplishes different assignments ranging from antigenic procession of molecules to activation of MMPs that are involved in the reconstruction of maternal endometrium (Riese *et al.* 1996, Mohamed & Sloane 2006). Song *et al.* (2006, 2007) reported an expression of CTSS in the endometrial stratum compactum, glandular, and luminal epithelial of ruminants. In this study, an obvious, but restricted increase of CTSS protein occurred

Table 2 Summary of selected results of the analysis of the flow cytometry for extracellular CD molecules versus intracellular targets (percentage of events with positive staining for proteins of interest).

	Average number of assigned cells per sample	CTSS	CST3	CST7	
$CD122^+$ $CD3^-$	372	44.9	90	61.54	Nonpregnant uteri
	3679	92.12	68.58	78.8	Decidua
$CD4^+$	507	74.4	10	60	Nonpregnant uteri
	567	63.46	8.5	0	Decidua
$CD8^+$	258	2.9	0	5	Nonpregnant uteri
	39	0	25	33.3	Decidua
$CD3^+$	2157	90.38	86	93.24	Nonpregnant uteri
	1113	86.4	85.95	90.98	Decidua

in the post-mated phase in mice, which was most likely achieved by the progesterone rise due to mating. Surprisingly, there was no *Ctss* mRNA expression in any of the single day 3 blastocysts examined by nested RT-PCR, contradicting the hypothesis of an active invasion of the embryo into the decidualized endometrium facilitated by CTSS. Immunohistochemical staining of blastocysts *in utero* supports this point of view. On the other hand, we detected the expression of *Il6* mRNA in nearly all single blastocysts. Therefore, in early murine pregnancy, IL6 might be part of an active blastocyst-derived paracrine activation of CTSS in maternal tissues, a hypothesis supported by a lack of CTSS-positive $CD8a^+$ dendritic cells herein. As found in this study, we can exclude $CD8a^+$ dendritic cells in pregnancy as a source for CTSS as a result of the flow cytometry analysis. Whereas uNKs, which are believed to play an essential part in the establishment of pregnancy, seem to be the major source for CTSS (Herington & Bany 2007). Additionally, studies in both CTSS- and CST3-null mice revealed angiogenic and neoplastic properties for CTSS in tumor nutrition and growth supporting the idea of CTSS being involved in embryo implantation, especially in the process of angiogenesis as observed but an increase in protein expression in the glands and of CST3 as an regulator of the depth of invasion, since tumor models are often used to imitate the processes of implantation (Wang *et al.* 2006). Concerning cathepsin proteases in general, CtsB and CtsL are well characterized in reproductive tissues and blastocysts (Song *et al.* 2007, Sireesha *et al.* 2008). Both of them were described as important factors in embryonic implantation and decidualization in mice (Afonso *et al.* 1997).

In response to the absence of CTSS in day 3 murine blastocysts in this study, these blastocysts were further examined upon a possible expression of the CTSS inhibitors, CST7 and CST3. The resulting RT-PCR fragments showed almost 100% expression of CST3 and 50% expression of CST7 mRNA in blastocysts examined, and furthermore, an intense staining of murine blastocysts was observed.

It was shown before that the embryonic expression of CST3 might be a possible hint for an apoptotic protection against cathepsins released during implantation, and additionally, a regulation of the expression of CTSB and CTSL in trophoblast giant cells via CST3 was reported (Afonso *et al.* 1997). Noteworthy, $CD8^+$ cells in the decidua seem to be a source for CST3 and CsF, whereas none of these $CD8^+$ cells could be assigned as CTSS positive.

The higher expression of CST3 in the estrous-dominated cycle might also indicate a maternal anti-invasive signal as the environment of the proliferative endometrium is not prepared to protect an embryo.

A further point of view that might be considered regarding embryonic implantation is the maternal acceptance of the embryo as a semi-allograft. Recently, the regulation of CTSS actions by CST7 in T-cells and other leukocytes was demonstrated (Hamilton *et al.* 2008). All of these cells immigrate in the early modulations of secretory phase maternal endometrium being part of the preparations for an embryo implantation. The decrease in $CD3^+$ cells detected via flow cytometry in this study might coincide with mechanisms regarding the maternal acceptance as mature T-cells, and especially, NK cells might lead to a rejection of embryonic implantation, whereas blastocyst-derived IL6 serves as a chemoattractant for the pro-invasive immunocompetent network (Fest *et al.* 2007).

The subsidiary crosstalk between embryonic cytokines and chemokines and the maternal response might be one of the keys to a successful implantation (van Mourik *et al.* 2009). Despite a lot of research in the last decade regarding the establishment of a receptive endometrium and attempts to optimize culture media and conditions for the embryo in assisted reproductive techniques (ART), many women still do not conceive or undergo an early miscarriage (Achache & Revel 2006, Sherwin *et al.* 2006, Gardner 2007, Arck *et al.* 2008). Hence, the baby-take-home rate in ART cycles still remains low (<20%; Deutsches IVF Register Annual Report 2006, p 10, http://www.deutsches-ivf-register.de/pdf-downloads/dirjahrbuch2006.pdf). The complexity and sensitivity of the mechanisms underlying the early feto-maternal dialogue demand for intense research in order to improve the outcome of ART techniques. The proteinase CTSS and its inhibitors take part in the agonist-inhibitor profile necessary for a proper implantation.

Materials and Methods

Animals

Planning and conduction of the experimental procedures as well as maintenance of the animals were carried out in accordance with the German Guide for the Care and Use of Laboratory animals and the ethics board of the Heinrich Heine University. Female, 6–8-week-old mice (*Mus musculus*) from the B6C3F1 strain were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA, USA) and maintained at 22–24 °C on a 12 h light:12 h darkness cycle. Female mice were superovulated by i.p. injection of 10 IU pregnant mare serum gonadotropin (PMSG, Sigma–Aldrich). Forty-eight hours after PMSG, ovulation was induced by i.p. injection of 10 IU human chorionic gonadotropin (hCG, Sigma–Aldrich). Female mice were impregnated by 12-week-old fertile males of the same strain. A single male was placed with two females overnight. Mating was verified by the appearance of a vaginal plug on the following morning.

Isolation of murine blastocysts

Mated mice were killed by cervical dislocation 96 h after hCG injection. Uteri were removed and flushed with IVF medium (Cleavage Medium; COOK Medical, Limerick, Ireland) under

visual control. Blastocysts were removed with a Pasteur pipette (Fisher Scientific, Schwerte, Germany) and placed in a droplet of IVF medium under oil (Reproline Medical GmbH, Rheinbach, Germany) and cultured at 37 °C in humidified air containing 5% CO_2. Single embryos and pools consisting of three blastocysts were transferred into thin wall PCR tubes (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) for further analysis of the mRNA expression of β-actin, *Ctss*, *Cst3*, *Cst7*, and *Il6* by RT followed by two rounds of PCR.

RT reaction

For each embryo, 18.2 μl RT-mastermix was prepared (2.5 mM $MgCl_2$, 1×RT buffer, dNTPs each 1 mM, 1.25 mM oligo $d(T)_{16}$, diethylpyrocarbonate (DEPC)-treated dH_2O ad 18.2 μl; all GeneAmp RNA PCR Reagent Kit, Applied Biosystems, Foster City, CA, USA), filled into a thin wall PCR tube with a single embryo in 1 μl media (Biozym Scientific GmbH) and kept on ice until RNA extraction. For RNA extraction, samples were heated up to 99 °C for 1 min in a Biometra T-Gradient (Biometra, Goettingen, Germany) to release the total RNA and denature the proteins. Samples were cooled down to 4 °C, and 10 U RNase inhibitor and 15 U MuLV RT (both Applied Biosystems) were added. The protocol for the RT reaction was as follows: 25 °C for 10 min, 42 °C for 30 min, 99 °C for 5 min, and 4 °C for ∞. After the reaction was complete, samples were diluted with DEPC-treated dH_2O ad 50 μl and stored at −20 °C until the PCR reaction was carried out.

DEPC-treated H_2O was used as a negative control and murine uteri cDNA as a positive control. The organs were isolated from C6B3F1 female mice and RNA extracted using a single-step method described by Chomczynski & Sacchi (1987).

Primers used for PCR

Sequences of cDNA clones for the mRNAs of interest in single mouse embryos (β-actin (accession number M12481), *Ctss* (AF038546), *Cst7* (NM_009977.2), *Cst3* (NM_009976), and *Il6* (NM_031168)) were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institute of Health (http://www.ncbi.nlm.nih.gov/Genbank). One set of corresponding outer primer sequences and one set of corresponding inner primer sequences were constructed using OLIGO 4.1 Primer Analysis Software (National Bioscience, Plymouth, MN, USA) and synthesized by the Eurofins MWG Operon (Ebersberg, Germany). To ensure that the product detected resulted from amplification of cDNA rather than contaminating genomic DNA, primers were designed to cross intron–exon boundaries. The primer cDNA sequences for and the sizes of the amplicon are listed in Table 3. Although the use of two nested primer pairs should yield in a high specificity for the amplified cDNA, we additionally confirmed the identity of the amplicons of *Ctss*, *Cst7*, *Cst3*, and *Il6* obtained from mouse uterus cDNA as a positive control as well as from blastocysts by sequence analysis. PCR products from the amplification of utero and embryonic cDNA with the specific primer sets were separated by agarose gel electrophoresis, and the amplicons were extracted with an agarose gel extraction kit (Macherey-Nagel, Düren, Germany). Extracted cDNA was cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced by the Biomedical Research Center (BMFZ) of the Heinrich Heine University, Duesseldorf, Germany. The respective identities were confirmed by comparison between the expected and extracted DNA sequences using BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

PCR

A total of 5 μl of diluted RT product was added to 45 μl of specific PCR-1-mix for β-actin, *Ctss*, *Cst3*, *Cst7*, and *Il6* (2 mM $MgCl_2$, 1×PCR buffer, 1× Q-solution, dNTPs each 0.2 mM, outer primer pair each 0.3 μM, 2.5 U/rct HotStarTaq Plus DNA polymerase, DEPC-treated dH_2O ad 45 μl; all Qiagen). After mixing all components in a thin wall PCR tube (Biozym Scientific GmbH), the reaction mixture was covered

Table 3 Primers used for RT-PCR and nested RT-PCR.

mRNA	Accession number of mRNA	Size of amplified fragment (bp)	Type of primer	Sequence of primer (5′–3′)
Ctss out	AF038546.1	660	5′	5′-cct acc aag tgg gca tga ac-3′
			3′	5′-gcc atc aag agt ccc ata gc-3′
Ctss in		240	5′	5′-taa tcg gac att gcc tga ca-3′
			3′	5′-ctg gaa agc ttc ggt cat gt-3′
Cst3 out	NM_009976	482	5′	5′-tcg ctg tga gcg agt aca ac-3′
			3′	5′-cat ggc agg tac tgc aag aa-3′
Cst3 in		206	5′	5′-tgg tga gag ctc gta agc ag-3′
			3′	5′-tgc agc tga att ttg tca gg-3′
Cst7 out	NM_009977.2	479	5′	5′-ggt cct gga gct gta ctt gc-3′
			3′	5′-aga gga gaa cag gca cct ca-3′
Cst7 in		89	5′	5′-atg cat cac caa ctg gac aa-3′
			3′	5′-ggg atg acc cag act tca ga-3′
Il6 out	NM_031168	339	5′	5′-gtt ctc tgg gaa atc gtg ga-3′
			3′	5′-gga aat tgg ggt agg ga-3′
Il6 in		226	5′	5′-tgt gca atg gca att ctg at-3′
			3′	5′-ctc tga agg act ctg gct ttg-3′
β-Actin	NM_12481	409	5′	5′-caa ggt gtg atg gtg gga atg g-3′
			3′	5′-cag gat ggc gtg agg gag agc a-3′

with light white mineral oil (Sigma–Aldrich), put in the T-Gradient (Biometra), and heated to 95 °C for 5 min to activate the hot-start enzyme. After completion of 40 cycles of 94 °C for 30 s, 54 °C (β-actin)/56 °C (*Ctss* and *Il6*)/57 °C (*Cst7*)/51 °C (*Cst3*) for 45 s, and 72 °C for 60 s, the reaction was terminated at 72 °C for 10 min and the reaction mixture was cooled down to 4 °C for ∞. First-round PCR products were stored at −20 °C until the second PCR was conducted.

For the second PCR, 5 μl of the first-round PCR products were added to 45 μl PCR-2-mix (2 mM $MgCl_2$, 1×PCR buffer, 1× Q-solution, dNTPs each 0.2 mM, inner primer-pair each 0.3 μM, 2.5 U/μl HotStarTaq Plus DNA polymerase, DEPC-treated dH_2O ad 45 μl; all Qiagen) in a thin wall PCR tube (Biozym Scientific GmbH) covered with white light mineral oil (Sigma–Aldrich) and put in the T-Gradient (Biometra). Program parameters were identical to first round protocol except the annealing temperature for CTSS with 60 °C. Samples were stored at −20 °C until agarose gel electrophoresis was carried out.

Agarose gel electrophoresis

Horizontal 2% agarose gel electrophoresis was carried out in the presence of ethidium bromide (0.5 μg/ml; Sigma–Aldrich), and HyperLadder II (Bioline, Luckenwalde, Germany) was used to determine the sizes of the amplified fragments. After completion of electrophoresis, the agarose gel was analyzed by applying the GelDoc 1000 system (Bio-Rad Laboratories). The cDNA size calculation and densitometry were carried out by using Molecular Analyst Software (Bio-Rad Laboratories).

Immunohistochemistry

Frozen sections of murine uteri in estrogen- and progesterone (after mating) – dominant phase, day 3 blastocysts *in utero*, and murine implantation sites (day 7 pc) were examined for the expression of the POI. Regarding the hormonal dependence of the murine cycle, progesterone occurrence is obtained only after mating. The cycle of the nonmated female mouse is estrogen dominated.

Sections were fixed in cold acetone for 10 min and washed with PBS, then incubated in 0.3% H_2O_2 for 20 min. According to the manufacturer's instructions for Vectastain Elite ABC Kits (Vector Laboratories, Burlingame, CA, USA), sections were incubated with diluted normal blocking serum from the species in which the secondary antibody was raised (anti-mouse and anti-goat) to avoid unspecific staining. Afterwards, sections were washed and incubated with primary antibody (1:100 rabbit anti-human pan-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); 1:10 rabbit anti-mouse CTSS (Santa Cruz Biotechnology); 1:50 goat anti-mouse CST7 (Santa Cruz Biotechnology); 1:12.5 goat anti-mouse CST3 (R&D systems, Wiesbaden-Nordenstadt, Germany)) for 1 h at room temperature. After washing with PBS, the appropriate biotinylated secondary antibody was applied, sections were rinsed and immunoperoxidase staining with diaminobenzidine tetrahydrochloride (Vector Laboratories) performed. Sections were dehydrated with graded ethanol concentrations (70–98%) and xylene, and were subsequently mounted with Mounting Media (Vector Laboratories).

Negative control experiments included incubation with the IgG fraction of normal host serum or secondary antibody alone. Staining with pan-cytokeratin served as control for ductal and glandular epithelial cells. The procedure was repeated two times for several sections (minimum of two) from each chosen sample (minimum of four).

The intensity of the immunohistochemical staining of the samples was rated by two independent investigators (Table 1).

Flow cytometry

Pooled samples of nonpregnant mice (uteri, $n=4$), decidua ($n=4$) from pregnant animals, and blood from 8-week-old female mice were obtained on day 9 pc.

Mononuclear cells from peripheral blood were isolated with LymphoPrepTM (Fresenius Kabi Norge AS for Axis-Shield Poc AS, Oslo, Norway) according to the manufacturer's instruction. Tissues were mechanically dissected, then enzymatically digested with 0.25% trypsin and 0.05% collagenase in PBS (Sigma–Aldrich), and placed at 37 °C for 1 h on an orbital shaker as described before (Marsh *et al.* 2008). For the isolation of the nuclear cells, tissues were strained with 100 μm mashes, cell numbers counted, centrifuged, and resuspended in PBS with 0.5% FBS. The staining for cytometrical analysis started with 10^5 cells per FACS tube (BD Pharmingen, Heidelberg, Germany).

First, the staining of extracellular targets was carried out with mouse T lymphocyte subset antibody cocktail (PE-Cy7 CD3e, PE CD4, and APC CD8a), FITC rat anti-mouse CD122, PE rat anti-mouse CD122, PE-Cy7 armenian hamster anti-mouse CD3 (all BD Pharmingen) according to the manufacturer's instruction regarding concentration. Cells were incubated for 20 min at 4 °C in the darkness, washed with PBS twice, and suspended in 0.1% formalin. Following this, the staining for the intracellular targets was done. Cells were fixed in 2% formalin at 37 °C for 10 min, permeabilized with 1% Tween 20 (Sigma–Aldrich) for 30 min, washed twice and incubated with rabbit anti-mouse CTSS (Santa Cruz), goat anti-mouse CST7 (Santa Cruz), and goat anti-mouse CST3 each 1:200 in 10% serum of the host species of the secondary antibody. After washing twice with PBS, cells were incubated with the secondary antibodies Alexa Fluor 568 anti-rabbit IgG and Alexa Fluor 488 and 594 anti-goat IgG each 1:4000 (Molecular Probes, Karlsruhe, Germany), washed, and fixed in 0.1% formalin.

The whole samples were acquired on a Cytomics FC 500 flow cytometer, and data were analyzed with the RXP software (both Beckman Coulter, Krefeld, Germany).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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2.3 CXCL1 expression in human decidua *in vitro* is mediated via the MAPK signalling cascade (submitted to Journal of clinical endocrinology and metabolism - under review)

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A proper decidualization as a part of the regular menstrual cycle in human is an important precondition for the establishment of a successful pregnancy. This process includes molecular and histological modifications in the maternal tissue enabling the invasion of specialized uterine natural killer cells (uNK) mediating the acceptance of the semi-allograft embryo as well as vasculogenesis and subsequent angiogenesis supporting embryonic nutrition and survival. The early embryo-maternal dialogue subsequently supports those modifications of the maternal endometrium and therewith ensures its own invasion. One of the early secreted products of the embryo mediating the embryos effect on the maternal decidua is the chemokine interleukin-1ß (IL-1ß). Recently, a study revealed the gene expression profile of human primary decidualized endometrial stromal cells in an *in vitro* co-culture model with trophoblast conditioned medium. One of the most upregulated genes was the chemokine ligand 1 (CXCL1). CXCL1 was first described as hormonally regulated during decidualization of endometrial stroma cells involved in leukocyte migration. So far it is known that chemokines containing an glutamic acid-leucine-arginine (ELR) motif within their amino acid sequence, such as CXCL1 and chemokine ligand 8 (CXCL8, IL-8), are involved in angiogenesis *in vivo* as well as *in vitro* besides their ability of neutrophil recruitment to inflammation sites. Angiogenesis and maternal acceptance of the semi-allograft embryo belong to the most important mechanisms in implantation and placentation, both processes in which decidual CXCL1 seems to be a central key player. Generally, the IL-1ß response in different cell types such as immunocompetent cells, endothelial and epithelial cells and fibroblasts comprises complex signalling networks and activation of MAPK, c-Jun N-terminal kinase (JNK) and NFκB. A lack of Signal Transducers and Activator of Transcription (STAT3) signalling was described as a potent factor of unexplained infertility during the window of implantation.

To investigate the maternal regulation of CXCL1 as one of the key player chemokines in the human decidua, IL-1ß, imitating early embryonic signalling, was used to investigate the involved signalling cascades in human decidualized endometrial stromal cells *in vitro*.

A total of 99% cells were positive for ß-actin mRNA expression and about 85% cells were positive for PRL mRNA expression. Only ß-actin and PRL positive cDNA samples were further examined for CXCL1 mRNA expression in concentration- and time-dependent manner. Regarding the concentration of the embryo surrogate, IL-1ß, the mRNA expression of CXCL1 increased from 0 to 10ng/ml during 24h, but the most prominent statistically significant increase was measured after 48h incubation with 0.1ng/ml IL-1ß. Hence, the time course experiment confirmed this result as the most intense mRNA expression was detected after 48h with 0.1ng/ml IL-1ß. The results on mRNA level representing the most intense CXCL1 signal after 48h with 0.1ng/ml IL-1ß could be confirmed on protein level. Furthermore, the protein secretion in the cell-culture supernatant increased after 24h and 48h from 0-10ng/ml IL-1ß, but again the concentration of 0.1ng/ml of the embryo´s surrogate marker IL-1ß for 48h was the only concentration leading to a statistically significant CXCL1 secretion of dES. First, the initiation of CXCL1 protein secretion by the embryo´s surrogate marker IL-1ß was proven by an approach using the IL-1ra. IL-1ra competes with IL-1ß for the binding to the IL-1 receptor type I (IL-1R1). Even low concentrations of IL-1ra (starting with 1ng/ml) decreased the CXCL1 secretion of dES. A concentration of 50ng/ml led to a statistically significant reduction of CXCL1 secretion of dES and a complete inhibition of CXCL1 secretion was achieved by applying > 50ng/ml of IL-1ra confirming the activation of decidual CXCL1 synthesis by IL-1ß.

Further approaches regarding IL-1ß mediated CXCL1 signalling in dES focussed on MAPK, STAT3 and JNK signalling. dES were pre incubated with varying non cytotoxic concentrations of the inhibitors for 2h followed by 48h incubation with 0.1ng/ml IL-1ß. Pre incubation with the MAPK inhibitor, PD 980592, led to a statistically significant decrease of 60% of the CXCL1 secretion in dES cell culture supernatant compared to controls beginning

at low concentrations of the inhibitor (25µM). Whereas, only a distinct but not statistically significant decline of 35% and 30%, respectively, of the CXCL1 secretion was observed using either 250µM of STAT3 or 100µM JNK inhibitor.

The synchronization of the molecular mechanisms between the preimplantation embryo and the maternal decidua is a major prerequisite for the establishment of a successful pregnancy. Chemokines such as CXCL1 are known to play a critical role in the early embryo maternal dialogue supporting invasion and implantation, maternal acceptance of the semi-allograft, angiogenesis and placentation. It is possible that endometrial CXCL1 on one hand might function as a chemoattractant for immune cells in order to induce and regulate maternal acceptance of the embryo. On the other hand, endometrial derived CXCL1 might also be involved in proper implantation of the embryo by supporting the homing and attachment of the pre implantation embryo as it was hypothesized before. Therefore, it was first shown in the current study that endometrial CXCL1 is a target gene of the embryos derived secretion product IL-1ß since the CXCL1 synthesis in dES can be completely inhibited by IL-1ra. The concentration of IL-1ra used for the inhibition of CXCL1 expression in dES is comparable to physiologic serum concentrations determined in a clinical study of injury. Data from human endometrium and especially epithelium are limited regarding concentration of IL-1ra. In human endometrial epithelial cells, prostaglandin E_2 and F_2 synthesis induced by IL-1α and IL-1ß could be inhibited *in vitro* by 30ng/ml IL-1ra or 90ng/ml IL-1ra, respectively. Our data show that MAPK activity, especially MEK-1 activity, seems to be the most important signalling cascade in mediating the embryos´ surrogate IL-1ß signal regarding CXCL1 expression in human dES *in vitro*.

The embryo maternal dialogue consists of a complex signalling network in the human decidualized endometrium *in vitro*. Within this complex network, MAPKinase is the major signalling cascade involved in regulating CXCL1 as a key player in the process of decidualization and implantation. Further knowledge about the factors taking part in the

embryo maternal dialogue, their signalling and cellular responses might contribute in treating infertility, improving ART outcomes and minimizing pregnancy associated pathologies.

Declaration of DM Baston-Büst´s role:

Experimental outline, RNA and isolation, reverse transcription and PCR, ELISA, interpretation of the data, writing of the manuscript

Main title

CXCL1 expression in human decidua *in vitro* is mediated via the MAPK signalling cascade

Abbreviated title

MAPK signalling mediates CXCL1 expression

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key terms: embryo-maternal dialogue, CXCL1, IL-1β, signalling cascades, human, decidualization

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The authors disclose any potential conflict of interest.

Embryo derived IL-1ß, a key component of the embryo-maternal dialogue, influences CXCL1 expression in maternal decidua via the MAPK signalling cascade.

Abstract

Context: Early molecular interaction between embryo and mother, involving chemoattractans, especially chemokine ligand 1 (CXCL1), determines the pregnancy outcome. So far nothing is known about the signalling cascades of CXCL1 expression in human decidua.

Objective: The aim of the study was to identify signalling cascades mediating the CXCL1 expression in human decidua incubated with IL-1ß as a major secretion product of the embryo.

Design: Decidualized endometrial stromal cells (dES) were incubated with IL-1ß in a concentration- and time-dependent manner. The specificity of the CXCL1 expression was verified by IL-1 receptor antagonist (ra) application and subsequent CXCL1 expression disappearance. IL-1ß signalling was examined using inhibitors for MAPKinase, Signal Transducers and Activator of Transcription (STAT3) and c-Jun N-terminal kinase (JNK) cascades by measurement of CXCL1 secretion.

Setting: Basic research.

Main outcome measures: We aimed to see an increase in mRNA- and protein-expression of CXCL1 after IL-1ß incubation and its inhibition on protein level after IL-1ra pre incubation. Pre incubation with the signalling cascade inhibitors ought to reveal the pathways involved in facilitating IL-1β action of CXCL1 expression.

Results: A statistical significant increase in CXCL1 mRNA- and protein-expression after incubation with 0.1ng/ml IL-1ß over 48 hours was detected. CXCL1 protein secretion was prevented by IL-1ra treatment. Only inhibition of the MAPKinase pathway resulted in a significant decrease of CXCL 1 protein expression.

Conclusions: CXCL1 is a target for the embryos secretion product IL-1ß in dES. IL-1ß signalling regarding CXCL1 synthesis is mediated via the MAPK-signalling cascade.

Introduction

A proper decidualization as a part of the regular menstrual cycle in human is an important precondition for the establishment of a successful pregnancy. This process includes molecular and histological modifications in the maternal tissue enabling the invasion of specialized uterine natural killer cells (uNK) mediating the acceptance of the semi-allograft embryo as well as vasculogenesis and subsequent angiogenesis supporting embryonic nutrition and survival (1-3). The early embryo-maternal dialogue subsequently supports those modifications of the maternal endometrium and therewith ensures its own invasion (4). One of the early secreted products of the embryo mediating the embryos effect on the maternal decidua is the chemokine interleukin-1ß (IL-1ß) (5).

Recently, a study revealed the gene expression profile of human primary decidualized endometrial stromal cells in an *in vitro* co-culture model with trophoblast conditioned medium (6). One of the most upregulated genes was the chemokine ligand 1 (CXCL1). CXCL1 was first described as hormonally regulated during decidualization of endometrial stroma cells involved in leukocyte migration (7). So far it is known that chemokines containing an glutamic acid-leucine-arginine (ELR) motif within their amino acid sequence, such as CXCL1 and chemokine ligand 8 (CXCL8, IL-8), are involved in angiogenesis *in vivo* as well as *in vitro* besides their ability of neutrophil recruitment to inflammation sites (8, 9). Angiogenesis and maternal acceptance of the semi-allograft embryo belong to the most important mechanisms in implantation and placentation, both processes in which decidual CXCL1 seems to be a central key player.

Although the knowledge about early embryonic factors influencing implantation increased in the last decade, little is known about the signalling cascades involved in regulating decidual factors such as CXCL1. Former primate studies revealed that IL-1ß mediates its role in decidualization of baboon endometrium via phosphorylation of MAPKinase and nuclear factor kappaB (NFκB) activity resulting in matrix metalloproteinase 3 activity and endometrial reorganization (10). Generally, the IL-1ß response in different cell types such as immunocompetent cells, endothelial and epithelial cells and fibroblasts comprises complex signalling networks and activation of MAPK, c-Jun N-terminal kinase (JNK) and NFκB (11). JNK signalling was previously detected in human secretory phase endometrium (12). Furthermore, a lack of Signal Transducers and Activator of Transcription (STAT3)

signalling was described as a potent factor of unexplained infertility during the window of implantation (13).

A better knowledge about those cascades could improve strategies and therewith outcome of assisted reproductive techniques (ART), which is urgently needed since the pregnancy rates still remeian unsatisfactory low although major improvements regarding therapy and culture conditions were made. To investigate the maternal regulation of CXCL1 as one of the key player chemokines in the human decidua, IL-1ß, imitating early embryonic signalling, was used to investigate the involved signalling cascades in human decidualized endometrial stromal cells *in vitro*.

Material & methods

Cell culture

The well characterized immortalized endometrial stromal cells - St-T1 (14, 15) - were cultured in DMEM/MCDB 105, 10% charcoal-stripped FBS, 1× penicillin/streptomycin, 2mM L-glutamine, 1× MEM non-essential amino acids (all Biowest, Nuancé, France), 6.6µg/ml insulin (Sigma-Aldrich, Taufkirchen, Germany). Cells were used at passages 3-6 in this study. Cells were decidualized after reaching confluency *in vitro* with 0.5mM cAMP, 1µM progesterone in insulin-free medium (DMEM/MCDB 105, 1× penicillin/streptomycin, 2mM L-glutamine, 50µg/ml ascorbic acid, 2% charcoal stripped FBS, 1× MEM non-essential amino acids, 5µg/ml transferrin) for 72 hours. The succesful decidualization was verified by prolactin (PRL) mRNA expression by PCR.

Experimental conditions

Decidualized cells were incubated with 0 - 10ng/ml recombinant human IL-1ß (R & D Systems, Minneapolis, MN, USA) for 0 - 96h in time- and concentration- dependent studies. Furthermore, decidualized cells were preincubated with inhibitors (IL-1ra [0-200ng/ml]; Promo Cell GmbH, Heidelberg, Germany; PD 980592 [0-500µM] - MAPKinase inhibitor -; STAT3 inhibitor peptide [0-750µM]; JNK-inihibitor I [≤ 10µM]; all Calbiochem, Darmstadt, Germany) for 2h followed by 48h of incubation with 0,1ng/ml IL-1ß.

Cell culture supernatant was collected and frozen, cell monolayers resuspended in peqGold TriFast (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and stored at -80°C up to further analysis. The procedures were repeated twice with quartets in each experimental group.

RNA extraction and reverse transcription (RT)

Total RNA was isolated after the single-step method described by Chomczynski & Sacchi (16). Cells were homogenized according to the manufacturers` protocol (PEQLAB). Equal amounts of RNA [2µg] were restricted with desoxyribonucleaseI (DNaseI) (Fermentas, Sankt Leon-Rot, Germany) before performing reverse transcription (RT) (High capacity cDNA RT Kit, Applied Biosystems Inc, Foster City, CA, USA) according to manufacturers` protocol (17).

Polymerase chain reaction (PCR) and primers

Sequences of cDNA-clones for the mRNAs that should be detected in decidualized cells (β-actin, PRL and CXCL1) were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH, Internet address: http://www.ncbi.nlm.nih.gov/sites/entrez) and synthesized by Eurofins MWG Biotech AG (Ebersberg, Germany). Primers were designed to cross intron/exon boundaries. PCR for ß-actin served as control for cDNA synthesis and only ß-actin positive samples were further examined for CXCL1 expression (data not shown). PCR was performed according to the manufacturers` instruction for DreamTaq™ Green DNA polymerase Master Mix (Fermentas) with 200ng cDNA as template. After completion, PCR-products were stored at - 20°C until 2% agarose-gel electrophoresis was carried out in the presence of ethidium bromide. After completion of electrophoresis, the agarose-gel was analyzed on the GelDoc 1000 system (Bio-Rad Laboratories, Hercules, CA, USA). The primer sequences and fragment sizes are listed in Table 1.

ELISA

CXCL1 as a mainly secreted protein was measured in cell-culture supernatant according to manufacturers` protocol (R & D Systems). Supernatant was diluted 44-times and measured in

duplicate. The minimum detectable CXCL1 concentration was less than 10pg/ml and the inter- and intra-assay coefficients of variation were < 10.2% and < 4.7%, respectively.

Statistical analysis

To investigate the statistical significance of the protein expressions, the Student´s t-test with $p<0.05$ and one way post-hoc ANOVA test with $p<0.05$ as cut-offs for statistical significance were carried out.

Results

RT-PCR

All samples were examined first for ß-actin mRNA expression. Furthermore, detection of PRL mRNA expression confirmed decidualization of dES. A total of 99% cells were positive for ß-actin mRNA expression and about 85% cells were positive for PRL mRNA expression (data not shown). Only ß-actin and PRL positive cDNA samples were further examined for CXCL1 mRNA expression in concentration- (Fig. 1) and time-dependent (Fig. 2) manner. Regarding the concentration of the embryo surrogate, IL-1ß, the mRNA expression of CXCL1 increased from 0 to 10ng/ml during 24h (Fig 1 A and B), but the most prominent statistically significant increase was measured after 48h incubation with 0.1ng/ml IL-1ß (Fig 1 A and B). Hence, the time course experiment confirmed this result as the most intense mRNA expression was detected after 48h with 0.1ng/ml IL-1ß (Fig. 2).

ELISA Analysis of CXCL1 Protein

Cell culture supernatants of ß-actin and PRL mRNA positive dES samples were measured for CXCL1 protein expression after 24 and 48h with 0-10ng/ml IL-1ß incubation by ELISA technique since CXCL1 belongs to the group of highly secreted proteins (Fig. 3). The results on mRNA level representing the most intense CXCL1 signal after 48h with 0.1ng/ml IL-1ß could be confirmed on protein level (Fig. 3). Furthermore, the protein secretion in the cell-culture supernatant increased after 24h and 48h from 0-10ng/ml IL-1ß, but again the concentration of 0.1ng/ml of the embryo´s surrogate

marker IL-1ß for 48h was the only concentration leading to a statistically significant CXCL1 secretion of dES. For further examination of the signalling cascades the concentration of 0.1ng/ml IL-1ß and an incubation time of 48h were applied.

IL-1ß signalling cascades influencing CXCL1 expression

First, the initiation of CXCL1 protein secretion by the embryo´s surrogate marker IL-1ß was proven by an approach using the IL-1ra. IL-1ra competes with IL-1ß for the binding to the IL-1 receptor type I (IL-1R1). Even low concentrations of IL-1ra (starting with 1ng/ml) decreased the CXCL1 secretion of dES (Fig. 4). A concentration of 50ng/ml led to a statistically significant reduction of CXCL1 secretion of dES and a complete inhibition of CXCL1 secretion was achieved by applying > 50ng/ml of IL-1ra confirming the activation of decidual CXCL1 synthesis by IL-1ß (Fig. 4).

Further approaches regarding IL-1ß mediated CXCL1 signalling in dES focussed on MAPK, STAT3 and JNK signalling. dES were pre incubated with varying non cytotoxic concentrations of the inhibitors for 2h followed by 48h incubation with 0.1ng/ml IL-1ß. Pre incubation with the MAPK inhibitor, PD 980592, led to a statistically significant decrease of 60% of the CXCL1 secretion in dES cell culture supernatant compared to controls beginning at low concentrations of the inhibitor (25µM) (Fig. 5). Whereas only a distinct but not statistically significant decline of 35% and 30%, respectively, of the CXCL1 secretion was observed using either 250µM of STAT3 or 100µM JNK inhibitor (Fig. 5).

Discussion

The synchronization of the molecular mechanisms between the preimplantation embryo and the maternal decidua is a major prerequisite for the establishment of a successful pregnancy. Chemokines such as CXCL1 are known to play a critical role in the early embryo maternal dialogue supporting invasion and implantation, maternal acceptance of the semi-allograft, angiogenesis and placentation (18, 19). CXCL1 mRNA was found among the most upregulated mRNAs in primary dES after contact with trophoblast conditioned media possibly taking part in stimulating the alteration of the immune microenvironment of the maternal decidua in favour of the embryonic implantation (6).

Furthermore, it was reported that CXCL1 mRNA of endometrial stromal fibroblasts incubated with uterine leukocytes is significantly increased and that the interaction of immune cells and endometrium might be important for the cyclic modifications of the endometrium and implantation (20). It is possible that endometrial CXCL1 on one hand might function as a chemoattractant for immune cells in order to induce and regulate maternal acceptance of the embryo. On the other hand, endometrial derived CXCL1 might also be involved in proper implantation of the embryo by supporting the homing and attachment of the pre implantation embryo as it was hypothesized before (21). Although an upregulation of CXCL1 mRNA was shown in cyclic endometrium and during decidualization and its importance in the process of implantation discussed elaborately, nothing is known about the signalling cascades mediating CXCL1 upregulation in the endometrium (7).

In the current study an immortalized endometrial cell line as a surrogate for the decidua and IL-1ß as a major secretion product of the trophoblast *in vivo* were used to investigate signalling cascades involved in embryo-maternal interaction since the i*n vitro* cell culture of human endometrium enables the research of these mechanisms during decidualization and early embryo contact which is impossible to investigate *in vivo* (15).

Therefore, it was first shown in the current study that endometrial CXCL1 is a target gene of the embryos derived secretion product IL-1ß since the CXCL1 synthesis in dES can be completely inhibited by IL-1ra. The concentration of IL-1ra used for the inhibition of CXCL1 expression in dES is comparable to physiologic serum concentrations determined in a clinical study of injury (22). Data from human endometrium and especially epithelium are limited regarding concentration of IL-1ra. In human endometrial epithelial cells, prostaglandin E_2 and F_2 synthesis induced by IL-1α and IL-1ß could be inhibited *in vitro* by 30ng/ml IL-1ra or 90ng/ml IL-1ra, respectively (23).

The activation of MAPK signalling which stimulates cell growth and differentiation seems to be important for the process of decidualization and implantation. Our data show that MAPK activity, especially MEK-1 activity, seems to be the most important signalling cascade in mediating the embryos´ surrogate IL-1ß signal regarding CXCL1 expression in human dES *in vitro*. It was shown in an immortalized cell line of extravillous trophoblast that incubation with similar concentrations of MEK-1 inhibitor PD 98059 could inhibit insulin-like growth factor binding protein 1 phosphorylation

of MAPK (24). Furthermore, Lathi *et al*. detected that signalling pattern in decidualized endometrium depends on the concentration of the ligand preferring MAPK at higher concentrations of insulin (25). So far, IL-1ß signalling was extensively examined in pro-inflammatory responses of the immune system (11). Herein, phosphorylation and ubiquitination of intracellular second messengers were found to activate MAPK and JNK pathways in order to regulate the expression of target genes such as the chemokine CXCL8 (11). Furthermore, it was reported that inflammatory IL-1ß induces CXCL1 expression in epithelial cells (HeLa) via MAPK activity stimulating migration of monocytes and macrophages (26). Studies from the baboon revealed that the embryonic IL-1ß stimulates phosphorylation of p38 MAPK within 10 minutes and activation of NF-κB during decidualization (10).

Regarding the activity of JNK, our data suggest that this pathway does not play a significant role in mediating the IL-1ß signal in dES. Albeit a tendency in CXCL1 reduction was observed when the JNK inhibitor was applied. The data of a previous study of cultured human endometrial stroma cells *in vitro* and immunohistochemistry *in vivo* displaying the highest JNK activity in secretory phase endometrium associated this activity with an inflammatory CXCL8 response (12). JNK activity was also found to inhibit the transcription of the progesterone receptor in human endometrial stromal cells being exposed to reactive oxygen species (27). Furthermore, JNK activity mediates mitogenesis of the human epithelial endometrial cell line Hec-1-A based on basic transcription element binding protein expression and resulting in growth factor synthesis (28).

Next to MAPK and JNK, cytokine and growth factor signalling can be mediated via the STAT3 pathway influencing cell survival, growth, transformation and differentiation of cells. All of these cellular aspects are important at the embryo maternal interface supporting endometrial reorganization, angiogenesis and invasion of trophoblastic cells (29). The data of the current study reveal that STAT3 also does not have a significant role in CXCL1 mediation by IL-1ß in dES. However, comparable to the results of JNK cascade inhibition observed in this study, a tendency in CXCL1 expression reduction via the STAT3 signalling cascade was detected. STAT3 signalling seems to be important during the window of implantation as a diminished level of STAT3 was found in the endometrium of women with unexplained infertility (13). These data are supported by a the mouse model, where a

lack of STAT3 activation by IL-11 and leukemia inhibitory factor inhibits the implantation of the embryo (30).

The embryo maternal dialogue consists of a complex signalling network in the human decidualized endometrium *in vitro*. Within this complex network, MAPKinase is the major signalling cascade involved in regulating CXCL1 as a key player in the process of decidualization and implantation. Further knowledge about the factors taking part in the embryo maternal dialogue, their signalling and cellular responses might contribute in treating infertility, improving ART outcomes and minimizing pregnancy associated pathologies.

Acknowledgement

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Tables, figures and legends

Table 1

Primer	Size of amplified fragment [bp]	Sequences 5´➔3´
ß-actin	239	5`- CGGGACCTGACTGACTACC 3`- AGGAAGGCTGGAAGAGTGC
PRL	247	5`- GCTTCTGTATCATCTGGTCACG 3`- TGCGTAGGCAGTGGAGCAG
CXCL1	194	5`- ATAGCCACACTCAAGAATG 3`- TCTGCAGCTGTGTCTCTCTT

Table 1: Primersequences for synthesis of cDNA clones of human β-actin, PRL and CXCL1.

Figure 1

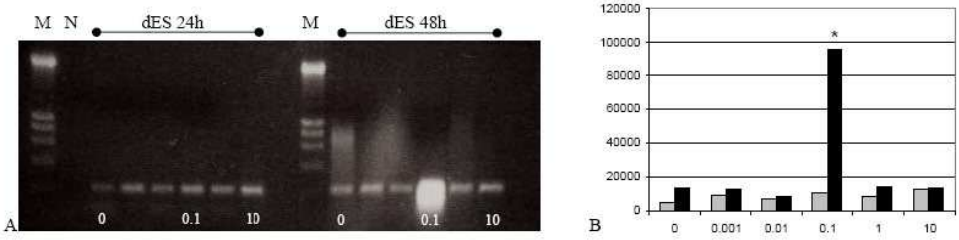
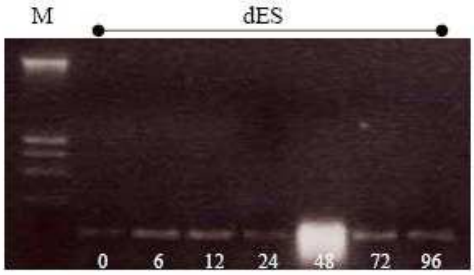


Figure 1: A) Representative electrophoresis of the expression of CXCL1 mRNA (size of the PCR product 194bp) in dES after 24 und 48h of IL-1ß incubation [0; 0.001; 0.01; 0.1; 1 und 10ng/ml], B) corresponding analysis of pixel density of CXCL1 PCR products after 24h (grey bars) and 48h (black bars) of IL-1ß incubation [0-10ng/ml]. Samples are shown following increasing concentrations of IL-1ß [0-10ng/ml]. M= marker of DNA band sizes; N= negative control.

Figure 2



275

Figure 2: Representative electrophoresis gel of the expression of CXCL1 mRNA (size of the PCR product 194bp) in dES after incubation with 0.1ng/ml IL-1ß for 0, 6, 12, 24, 48, 72 and 96h.

13

Figure 3

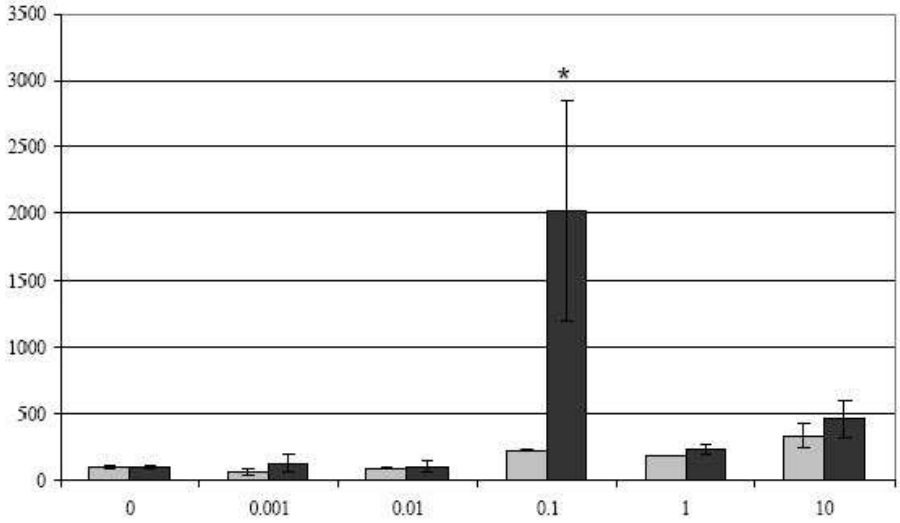


Figure 3: CXCL1 protein secretion [% relative to the value of samples of 0h as 100%] in cell culture supernatant of dES after incubation with different concentrations of IL-1ß [0-10ng/ml] for 24h (light grey bars) and 48h (dark grey bars) with +/- SEM. The asterisk (*) indicates a statistical significant difference with $p<0,05$.

Figure 4

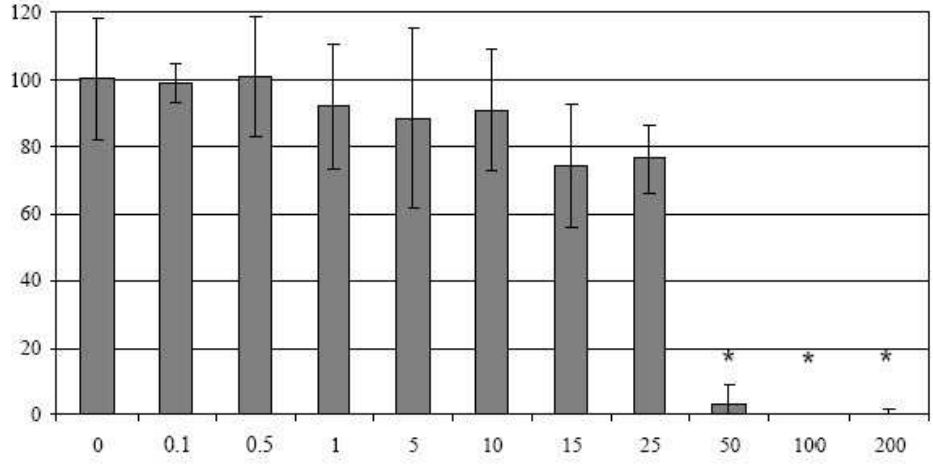


Figure 4: CXCL1 protein secretion [% relative to samples of 0ng/ml IL-1ra] in dES cell culture supernatant after 2h pre incubation with IL-1ra [0-200ng/ml] followed by 48h 0.1ng/ml IL-1ß with +/- SEM. The asterisk (*) indicates a statistical significant difference with $p<0.05$.

Figure 5

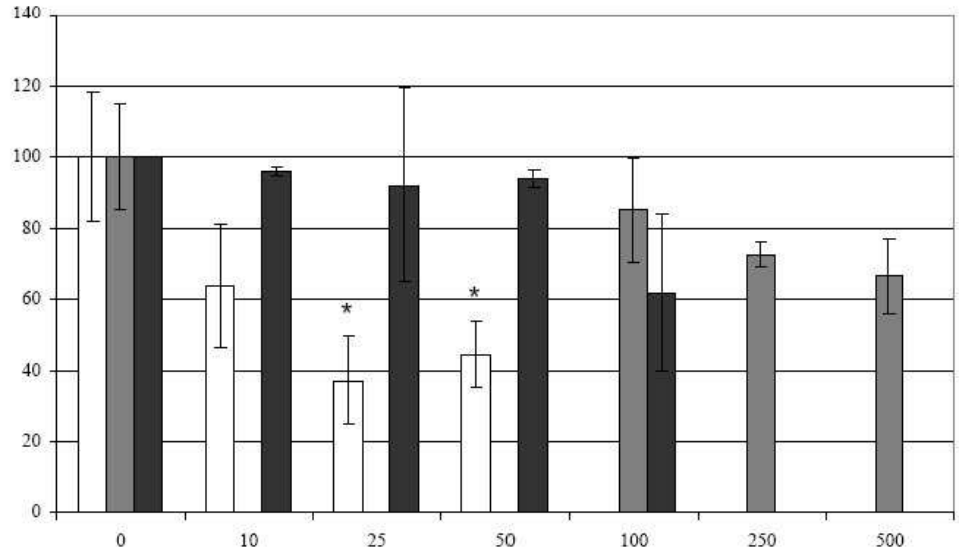


Figure 5: CXCL1 protein secretion [% in relation to the samples of 0µM of each inhibitor] in dES cell culture supernatant after 2h pre incubation with either MEK1- (white bars), STAT3- (light grey bars) or JNK- (dark grey bars) inhibitors [µM], followed by 48h of 0.1ng/ml IL-1ß incubation with +/- SEM. The asterisk (*) indicates a statistical significant difference with $p<0.05$.

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2.4 Syndecan-1 knock-down in decidualized human endometrial stromal cells leads to significant changes in cytokine and angiogenic factor expression patterns (submitted to Reproductive Biology and Endocrinology - under review)

DM Baston-Büst, M Götte, W Janni, JS Krüssel, AP Hess

The successful establishment of a pregnancy in human depends on a synchronized dialogue of maternal and fetal factors enabling attachment of the hatched embryo to the uterine wall. Invasion into the decidualized maternal endometrial stroma is followed by induction of angiogenesis and acceptance of the semi-allograft embryo by the maternal immune system. Interleukin-1beta (IL-1β) was identified as a secretion product of human syncytiotrophoblast and trophoblast in early human pregnancy. Furthermore, higher levels of IL-1β protein and chemokine ligand 1 (CXCL1) were shown to be expressed in the decidua of early pregnancy. CXCL1 plays an important role in mediating the acceptance of the maternal immune system towards the semi-allograft embryo by attracting specialized leukocyte populations, such as uterine natural killer cells (uNK), granulocytes and macrophages supporting essential modifications regarding implantation and protection of pregnancy. Especially CXCL1 was shown to be involved in early maternal reactions of the decidualized stroma to embryonic secretion products. This was depicted by a significant upregulation of *cxcl1* gene expression after coincubation with trophoblast-conditioned medium *in-vitro* as well as in a co-culture model of primary endometrial cells and trophoblast explants. Besides the signalling through the classical G-protein coupled receptors, the heparan sulfate proteoglycan syndecans (Sdc) also take part as co-receptors in mediating chemokine function by enhancing the binding of chemokines to their innate receptors. Syndecans are localized on the cell-surface and in the extracellular matrix. They consist of an ectodomain containing consensus sequences for heparan sulfate or chondroitin sulfate attachment, a single conserved transmembrane domain and a short cytoplasmic domain. Furthermore, they are described as multifunctional molecules in human, localized nearly ubiquitously and involved in wound healing, tumour growth, immune cell function and angiogenesis. Lately, the mRNA expression of Sdc-1 to -4

in human endometrium of normal cycling healthy women was observed with a prominent up regulation of Sdc-1 and -4 in whole tissue endometrium samples of secretory phase. Sdc-1 was also found in uteroplacental units in human, localized apical in chorionic villi actively invading maternal decidua, supposedly being involved in fetal-maternal interaction. We hypothesize that Sdc-1 plays an important role in the process of human decidualization and implantation by regulation of chemokine and angiogenic factor secretion in decidualized endometrial stroma cells supporting a proper embryonic attachment and subsequent implantation. In order to investigate this hypothesis, we generated a stable and inducible human endometrial stroma Sdc-1 knock-down cell line (called KdS1) which was further characterized on protein level by dot blot analysis of cytokine and angiogenic factor expression profiles. Furthermore, we investigated decidualized Sdc-1 knock-down cells (dKdS1) versus decidualized endometrial stroma cells (dSt-T1) after coincubation with the trophoblast secretion product IL-1β to identify Sdc-1´s role and expression profile changes in the implantation process.

The nuclear transfection of Tet(R) - coding plasmid pcDNA6/TR® was proven and evaluated via intensity rating of immunocytochemistry staining by two independent investigators (Figure 1). All six clones resulting from blasticidin selection were tested and the clone showing the strongest staining intensity for Tet(R) was selected for further transfection with Sdc1 shRNA.

The induction of Sdc1-RNAi in KdS1 with different tetracycline concentrations (0-1000 ng/ml) was tested for 24 and 48h and Sdc1-mRNA was measured quantitatively by RT-PCR. A 10^6-fold reduction of Sdc1-mRNA was detected after incubation with 1000 ng/ml Tet for 48h compared to untreated KdS1.

KdS1 cells were incubated with Tet for 48 h prior to 72h of decidualization protocol and tested for the expression of common decidualization markers IFGBP1 and PRL. dSt-T1 served as controls. The mRNA expression of PRL and IGFBP-1 showed no differences in dKdS1 versus dSt-T1.

Dot blot arrays for cytokines and angiogenic factors were performed after 72h decidualization of dKdS1 vs. dSt-T1 which served as controls.

The chemokines CXCL1, CXCL8, IL-6 and macrophage migration inhibitory factor (MIF) were secreted at significantly higher levels in dKdS1. Comparable amounts of IL-13 were secreted from dKdS1 and dSt-T1.

The expression of several cytokines was restricted only to dKdS1. Secretion of CCL2, CCL5, CXCL10, CXCL11 and soluble intercellular adhesion molecule (sICAM-1) occurred in dKdS1 supernatant only. Furthermore, we detected the peptide hormone granulocyte colony-stimulating factor (G-CSF) and the glycoprotein granulocyte macrophage colony-stimulating factor (GM-CSF), both involved in immune cell differentiation and infection mechanisms, only in dKdS1.

When investigating angiogenic factors after decidualization, dKdS1 supernatant contained significantly more hepatocyte growth factor (HGF, scatter factor), a paracrine factor which stimulates mitogenesis, angiogenesis and tumorigenesis, and pentraxin 3 (PTX3), a soluble molecule which belongs to the innate immune system. On the other hand, secretion of tissue inhibitor of metalloproteinases 1 (TIMP-1) and thrombospondin 1 (TSP1), both components of the extracellular matrix, was significantly increased in dSt-T1.

The secretion levels of the angiogenic factors angiopoietin-1 (Ang-1), angiogenin, IGFBP-2 and -3, matrix metalloproteinase 9 (MMP 9) and urokinase-type plasminogen activator (uPA) were similar in dKdS1 versus controls. CXCL16 could be identified to be exclusively expressed in dKdS1. Furthermore, dot blot arrays for cytokines and angiogenic factors were performed after 72h decidualization followed by 48h of coincubation with IL-1β imitating early embryonic contact in dKdS1 vs. the control dSt-T1.

Secretion of the cytokine MIF was significantly increased in dKdS1, whereas CXCL-8 secretion was higher in dSt-T1 cell culture supernatant. The secretion levels of CXCL1 and IL-6 were similar in dKdS1 and dSt-T1 after IL-1ß stimulation. Several cytokines associated with inflammatory mechanisms were only secreted in dKdS1 supernatant, namely G-CSF, GM-CSF, sICAM-1 and the chemokines CCL2, CCL5, CXCL10 and CXCL11. IL-13 was secreted in dSt-T1 exclusively.

Obvious changes also occurred regarding the expression profile of angiogenic factors after embryo imitating IL-1ß contact. HGF and IGFBP-2 were secreted significantly higher in dKdS1 compared to dSt-T1. IGFBP-3, TIMP-1 and TSP1 dominated in dSt-T1 supernatant vs. dKdS1. There were no statistically significant differences for MMP9, PTX3 and uPA in dKdS1 vs. dSt-T1. Ang-1 was exclusively secreted from dSt-T1 and angiogenin and CXCL16 were restricted to dKdS1.

We have demonstrated an increase in chemokine and angiogenic factor secretion upon Sdc-1 knock-down in decidualized human endometrial stroma cells. These results are consistent with the role of Sdc-1 as a co-receptor and storage factor for these molecules allowing new insights in interactions and regulations between chemokines and angiogenic factors contributing to an improved understanding of intermolecular network at the fetal-maternal interface. Removal of Sdc-1 from endometrial stroma cell surfaces by the process of ectodomain shedding or down regulation of its expression leads to a down regulation of binding sites for chemokines and angiogenic factors. As discussed in the following paragraphs, the nuclear factor-kappaB (NFκB) signalling cascade seems to be involved in Sdc-1 mediated processes in the human endometrium.

The precise molecular actions of Sdc-1 underlying the morphological and structural changes during the menstrual cycle in preparation for embryo implantation or menstrual shedding in absence of fertilization are unknown until today. This study provides the first evidence of an important contribution of Sdc-1 in regulating chemokine and growth factor action during the decidualization and implantation processes.

The glutamic acid-leucine-arginine (ELR) motif-positive chemokines CXCL1 and CXCL8 were secreted at higher levels in the Sdc-1 knock-down cells following decidualization, as compared to controls. Regarding secretion after contact with the embryo surrogate, IL-1ß, CXCL1 expression was comparable in both groups, whereas CXCL8 increased significantly in dSt-T1 supernatant compared to dKdS1. ELR-positive CXC-chemokines display angiogenic abilities, whereas chemokines lacking the ELR-motif are often characterized as

angiostatic factors. CXCL1 and CXCL8 bind to CXC-receptor 1 (CXCR1) and CXCR2, respectively. Sdc-1 was shown to interact with CXCL1, CXCL8 and their receptors via its anionic heparan sulfate chain. C-C motif chemokine ligand 2 (CCL2, formerly monocyte chemotactic protein-1, MCP-1) was described to be secreted into the uterine lumen as well as in the endometrial stroma by human primary endometrial epithelial cells and in first trimester decidua tissue functioning as a key player of monocyte chemotaxis Since we detected CCL2 only in dKdS1 supernatant upon decidualization and imitation of embryo contact, we propose an active role for Sdc-1 in binding of CCL2 in human decidualized endometrium *in vitro*. This storage of CCL2 by Sdc-1 in dES *in vitro* seems to be inconsistent with the *in vivo* situation of early pregnancy. Hence, there might be a temporal regulation of CCL2 expression as these data reflect the very early fetal-maternal dialogue. The absence of CCL2 expression in dES *in vitro* might result from the absence of factors that influence the decidua *in vivo*.

The secretion of CXCL10 and 11 might result from the lack of Sdc-1 in dKdS1 cell membranes. In dSt-T1, these molecules are supposingly stored at the cell membrane via Sdc-1 inhibiting early secretion in the supernatant. A synchronized pattern of angiogenic factors, growth factors and their inhibitors is therefore a critical precondition supporting not only angiogenesis but also modification of the extracellular matrix, decidualization and re-organization of the endometrial stroma.

Sdc-1 has been assigned as a HGF co-receptor intensifying its signalling via c-Met. The significant increase of HGF in dKdS1 supernatant after decidualization and after IL-1ß contact as seen in this study, suggests an intense interaction between Sdc-1 and HGF. It seems that Sdc-1 is important in HGF-binding and presentation towards its receptor for supporting cell proliferation and angiogenesis during the implantation period. We suggest that the lack of TSP1 secretion in dKdS1 is based on the absence of the known NF-κB activation by IL-1β in dKdS1. Therefore, the absence of TSP1 might lead to angiogenic malformation and dysregulation of the decidualized endometrium possibly leading to implantation failure.

Matrix metalloproteinases (MMPs) play an important role during the implantation phase regulated by their inhibitors, e.g. tissue inhibitor of metalloproteinase 1 (TIMP1), localized in fetal and maternal tissues. TIMP1 is the most secreted factor in dSt-T1 after decidualization and IL-1β stimulation. The knock-down of Sdc-1 in dKdS1 might therefore lead to a dysregulation of the TIMP-1 expression. The modulation of the maternal immune system enabling an embryonic invasion is one of the key processes of the early fetal-maternal communication. The important role of cytokines, like IL-1ß system, influencing decidualization- and implantation-related molecules, like MMPs, has already been reported. A recent study examined the components of endometrial secretions aspirated prior to embryo transfer in IVF and ICSI cycles, revealing the presence of pro-, as well as anti-inflammatory immuno-associated molecules. The pro-inflammatory cytokine IL-6 belonged to the main secretion products. In the present study, IL-6 secretion significantly increased in dKdS1 after decidualization. The increase in IL-6 secretion might be due to the lack of Sdc-1 and displays a potential intermolecular interaction between IL-6 and Sdc-1. These data correlate well with observations in Sdc-1 deficient mice subjected to kidney and allergic inflammation where an increase of IL-6 was observed.

The present study therefore underscores the importance of Sdc-1 as a co-receptor and a storage molecule in receptive endometrium supporting a proper biochemical foundation for embryonic implantation. Further investigation of Sdc-1s role in the process of implantation is needed, since its synchronous regulation of multiple receptor-dependent pathways seems to be a pivotal point in understanding and possibly ameliorating human implantation.

Declaration of DM Baston-Büst´s role:

Experimental outline, RNA isolation, reverse transcription and PCR, immunocytochemistry, dot blot analysis, interpretation of the data, writing of the manuscript

Title

Syndecan-1 knock-down in decidualized human endometrial stromal cells leads to significant changes in cytokine and angiogenic factor expression patterns

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Abstract

Background

Successful embryonic implantation depends on a synchronized embryo-maternal dialogue. Chemokines, such as chemokine ligand CXCL1, play essential roles in the maternal reproductive tract leading to morphological changes during decidualization, mediating maternal acceptance towards the semi-allograft embryo and induction of angiogenesis. Chemokine binding to their classical G-protein coupled receptors is essentially supported by the syndecan (Sdc) family of heparan sulfate proteoglycans. The aim of this study was to identify the involvement of Sdc-1 at the embryo-maternal interface regarding changes of the chemokine and angiogenic profile of the decidua during the process of decidualization and implantation in the human endometrium.

Methods

A stable Sdc-1 knock-down was generated in the immortalized human endometrial stromal cell line St-T1 and was named KdS1. The ability of KdS1 to decidualize was proven by Insulin-like growth factor binding 1 (IGFBP1) and prolactin (PRL) confirmation on mRNA level before further experiments were carried out. Dot blot protein analyses of decidualized knock-down cells vs non-transfected controls were performed. In order to imitate embryonic implantation, decidualized KdS1 were then incubated with IL-1ß, an embryo secretion product, vs controls. Statistical analyses were performed applying the Student´s t-test with $p<0.05$, $p<0.02$ and $p<0.01$ and one way post-hoc ANOVA test with $p<0.05$ as cut-offs for statistical significance.

Results

The induction of the Sdc-1 knock-down revealed significant changes in cytokine and angiogenic factor expression profiles of dKdS1 vs decidualized controls. Incubation with embryonic IL-1ß altered the expression patterns of KdS1 chemokines and angiogenic factors towards inflammatory-associated molecules and factors involved in matrix regulation.

Conclusions

Sdc-1 knock-down in human endometrial stroma cells led to fulminant changes regarding cytokine and angiogenic factor expression profiles upon decidualization and imitation of embryonic contact. Sdc-1 appears to play an important role as a co-receptor and storage factor for many cytokines and angiogenic factors during decidualization and the implantation period, supporting proper implantation and angiogenesis by regulation of chemokine and angiogenic factor secretion in favour of the implanting embryo.

Background

The successful establishment of a pregnancy in human depends on a synchronized dialogue of maternal and embryonic factors enabling attachment of the embryo to the uterine wall. Invasion into the decidualized maternal endometrial stroma is followed by induction of angiogenesis and acceptance of the semi-allograft embryo by the maternal immune system.

Cytokines are well-characterized factors in the implantation period [1]. Interleukin-1beta (IL-1β) was identified as a secretion product of human syncytiotrophoblast and trophoblast in early human pregnancy [2]. Furthermore, higher levels of IL-1β protein and chemokine ligand 1 (CXCL1) were shown to be expressed in the decidua of early pregnancy [3]. CXCL1 plays an important role in mediating the acceptance of the maternal immune system towards the semi-allograft embryo by attracting specialized leukocyte populations, such as uterine natural killer cells (uNK), granulocytes

and macrophages supporting essential modifications regarding implantation and protection of pregnancy [3, 4]. Chemokines belong to a subfamily of cytokines assigned by the position of conserved cysteins in their amino acid sequence. They function as chemoattractants for immunocompetent cells like leukocytes, which migrate towards the highest concentration of the chemoattractant, and act via G-protein coupled, 7 transmembrane-domains containing receptors on their target cells [5]. Especially CXCL1 was shown to be involved in early maternal reactions of the decidualized stroma to embryonic secretion products. This was depicted by a significant upregulation of *cxcl1* gene expression after coincubation with trophoblast conditioned medium *in-vitro* as well as in a co-culture model of primary endometrial cells and trophoblast explants [6, 7]. Besides the signalling through the classical G-protein coupled receptors, the heparan sulfate proteoglycan syndecans (Sdc) also take part as co-receptors in mediating chemokine function by enhancing the binding of chemokines to their innate receptors [8]. Sdcs are localized on the cell-surface and in the extracellular matrix. They consist of an ectodomain containing consensus sequences for heparan sulfate or chondroitin sulfate attachment, a single conserved transmembrane domain and a short cytoplasmic domain. Furthermore, they are described as multifunctional molecules in human, localized nearly ubiquitously and involved in wound healing, tumour growth, immune cell function and angiogenesis [9, 10]. Lately, the mRNA expression of Sdc-1 to -4 in human endometrium of normal cycling healthy women was observed with a prominent up regulation of Sdc-1 and -4 in whole tissue secretory phase endometrium samples [11]. Sdc-1 was also found in uteroplacental units in human, localized apical in chorionic villi actively invading maternal decidua, supposedly being involved in embryo-maternal interaction [12].

We hypothesize that Sdc-1 plays an important role in the process of human decidualization and implantation by regulation of chemokine and angiogenic factor secretion of decidualized endometrial stroma cells supporting a proper embryonic attachment and subsequent implantation. In order to investigate this hypothesis, we generated a stable and inducible human endometrial stroma Sdc-1 knock-down cell line (called KdS1) which was further characterized on protein level by dot blot analysis regarding its cytokine and angiogenic factor expression profile. Furthermore, we investigated decidualized KdS1 (dKdS1) vs decidualized endometrial stroma cells (dSt-T1) after coincubation with

the trophoblast secretion product IL-1β to identify Sdc-1´s role and expression profile changes in the process of decidualization and implantation.

Methods

Cell line and cell culture

The human endometrial stroma cell line St-T1 used in this study was a generous gift from Professor Brosens (Imperial College, UK, Great Britain). These cells were initially isolated from normal proliferative endometrial tissue during diagnostic laparoscopy, immortalized, named St-T1 and characterized for functionality and comparability to primary endometrial stromal cells before [13, 14]. They were maintained in a mixture of ¾ (v/v) DMEM and ¼ (v/v) MCDB 105, supplemented with 10% (v/v) charcoal-stripped fetal bovine serum (FBS), 1× penicillin/streptomycin, 40µg/ml gentamycin, 5µg/ml insulin (Sigma-Aldrich, Steelze, Germany), 2mM L-glutamine, 1mM non-essential amino acids and 1× sodium pyruvate (all except insulin Biowest, Nuaillé, France).

Transfection of pcDNA6/TR© via electroporation

The TREx™-system with the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit (Invitrogen, Karlsruhe, Germany) was chosen for stable, tetracycline (Tet) inducable Sdc-1 knock-down in immortalized St-T1. Therefore the plasmid pcDNA6/TR©, coding for the Tet-repressor (TetR), was transfected first in order to generate a TetR stable expressing host cell line. One cell clone expressing high levels of TetR was chosen as host for the inducible knock-down of Sdc-1 regulating the expression of the short hairpin (sh) RNA of Sdc-1 in trans (Figure 1). The circular plasmid pcDNA6/TR© was transfected succesfully in St-T1 using Nucleofection® in a Nucleofector® I (Lonza Cologne AG, Cologne, Germany). 10^6 cells were transfected per well with 3µg plasmid DNA in buffer V (Lonza Cologne AG) using the following programs: T-13, -23, -24, U-17, -23 and -24. The selection with 5µg/ml blasticidin started 48h after transfection was performed.

Immunocytochemistry for TetR

Transfected cells were tested for succesful nuclear transfection of TetR by immunostaining with mouse anti-TetR (500µg/ml) (MoBiTec, Göttingen, Germany). Controls were stained with non-specific mouse immunoglobulin (IgG) (200µg/ml) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Cells were cultured with adequate antibiotics on Nunc™ Lab-Tek® glass chamber slides (Thermo Scientific Fisher, Langenselbold, Germany), fixed and permeabilized with cold acetone according to manufacturer´s instructions for Vectastain® ABC-staining using an immunoperoxidase procedure (Vector Laboratories, Burlingame, CA, USA). Briefly, intracellular activity of peroxidases was quenched with 0.3% (v/v) H_2O_2 for 20min and cells consecutively incubated with blocking serum for 1h followed by incubation with 1st antibody (1:200 in blocking serum) for 1h. After incubation with the matching biotinylated 2nd antibody, cells were incubated with ABC reagent and staining visualized by incubation with peroxidase substrate solution (DAB, Vector). Cells were washed, dehydrated, coverslipped and photographed with a Leica DC 300F microscope (Leica, Solms, Germany). The clone with the most intense staining for TetR was chosen for further transfection (Figure 1).

Design of short hairpin RNAs

In a second step, short hairpin (sh) RNAs for the Sdc-1 knock-down were designed imitating tested transient silencer (si) RNAs for Sdc-1 (database sequence NM_002997) [15] using Invitrogen´s RNAi Designer (www.invitrogen.com/rnai) following the manufacturer´s instructions. The first four bases of the shRNA sequence are required for directed cloning in the vector pENTR™/H1/TO (see Table 1).

Cloning of shRNAs of interest in pENTR™/H1/TO and sequencing

For ligation in the pENTR vector, ds oligo and vector were mixed in a molar ratio of 50:1 and ligation performed according to manufacturer´s manual. Positive clones were analyzed by culturing 10 colonies each, followed by plasmid DNA isolation (GeneJET™ Plasmid Miniprep Kit; Fermentas, St. Leon-Rot, Germany) and sequencing with H1 forward and M13 reverse primers (Invitrogen) at the biomedical research center of the Heinrich-Heine University (Düsseldorf, Germany).

Transfection of pENTR™/H1/TO cloned fragments in pcDNA6/TR© transfected St-T1, selection

Clones identified with correct inserted shRNAs for Sdc-1 - one from each pair (Table 1) - were transfected into the TetR expressing host cell line via Nucleofection® with program T-23 as described above. Selection with 200µg/ml Zeocin® and 5µg/ml Blasticidin started 48h after Nucleofection®.

Primers for Polymerase Chain Reaction (PCR)

Sequences for β-actin, insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin (PRL) mRNAs were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH, Internet address: http://www.ncbi.nlm.nih.gov/sites/entrez). Primers were synthesized by Eurofins MWG (Ebersberg, Germany). To ensure that the product detected resulted from amplification of cDNA rather than contaminating genomic DNA, primers were designed to cross intron/exon boundaries. Furthermore, PCR products were sequenced at the biomedical research center of the Heinrich-Heine University (BMFZ) (Düsseldorf, Germany) and confirmed. The sequences and the sizes of the amplified fragments are listed in Table 2.

RNA isolation, reverse transcription, PCR and real-time PCR

Total RNA was isolated from cells applying the single-step method described by Chomczynski & Sacchi [16] and processed as described before [17]. Prior to reverse transcription (RT), DNA-free RNA was generated by a desoxyribonuclease I (DNase I) (Fermentas, St. Leon-Rot, Germany) digestion [18] as described before [19]. RT reaction was performed using 2µg RNA according to manufacturer´s instruction for High Capacity cDNA archive kit (Applied Biosystems Inc, Foster City, CA, USA).

In subsequent PCRs, β-actin was used as a housekeeping gene and reactions consisted of 1× DreamTaq™ Green PCR Master Mix (Fermentas), 120ng cDNA, 0.3µM forward and reverse primer and dH_2O ad 25µl. After completion of 35 cycles of 94°C for 1min, 94°C for 30sec, 56°C (β-actin and IGFBP-1) and 53°C (PRL) for 45sec and 72°C for 60sec, the reaction was terminated at 72°C for 7min and cooled down to 6°C in a peqSTAR 96 universal gradient thermocycler (PEQLAB Biotechnology,

Erlangen, Germany). PCR-products were stored at -20°C until 2% agarose-gel electrophoresis was carried out in the presence of ethidiumbromide (0.2µg/ml). After completion of electrophoresis, the agarose-gel was analyzed by the GelDoc 1000 system (Bio-Rad Laboratories, Hercules, CA, USA).

The quantitative PCR (real-time PCR) for IGFBP-1 normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the Qiagen QuantiTect SYBR Green PCR kit in a LightCycler (Roche, Indianapolis, IN, USA) as described previously [20]. Amplification specificity was verified using melting curve analysis and 2% agarose gel electrophoresis of the PCR products. Primer sequences are listed in Table 2.

The quantitative PCR for Sdc-1 was performed using TaqMan technology (Applied Biosystems Inc). cDNA corresponding to 50ng total RNA was used as a template in the PCR reaction consisting of ABI MasterMix (Applied Biosystems), and pre-designed TaqMan gene expression systems (Applied Biosystems) according to the manufacturers` instructions. For detection of Syndecan-1, primer Hs00174579_m1 was used and normalized to the expression of mammalian 18S ribosomal RNA (rRNA) (Hs99999901_s1, all primers by Applied Biosystems). RT-PCR was performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems) by using the default thermal cycling conditions (10min at 95°C, and then 40 cycles of 15sec at 95°C plus 1min at 60°C). Data were analyzed using the comparative C_t ($2^{-\Delta\Delta Ct}$) method [21].

Tetracycline induction of Sdc-1 knock-down in double transfected St-T1

The clone resulting from pair 1 was tested for Tet-induction according to manufacturers` instructions from 0 to 1000ng/ml Tet for 24 and 48 hours and analyzed by RT-PCR for Sdc-1 mRNA expression. The mRNA expression decreased 10^6-fold after 48h with 1000ng/ml Tet (data not shown). Subsequently, for further investigations an incubation time of 48h with 1000 ng/ml Tet was used. The resulting clone was named knock-down Sdc-1 (KdS1).

Decidualization of St-T1 and Tet-induced KdS1

St-T1 endometrial stroma cells (St-T1) are known to decidualize when treated with cyclic-AMP (0.5mM) and progesterone (1µM) for 72h [13, 14]. Double transfected and Tet-induced KdS1 cells

were decidualized under the same conditions to investigate possible differences in their ability to decidualize due to the Sdc-1 knock-down. Decidualization was verified by carrying out a RT-PCR for the known decidualization markers IGFBP-1 and PRL.

Investigation of chemokine and angiogenic factor profiles via dot blot analysis with cell culture supernatant of decidualized St-T1 and KdS1 with or without IL-1β incubation

dSt-T1 and dKdS1 (passages 3-6) were tested for IGFBP1 and PRL mRNA expression as described above. Cells were then incubated with or without IL-1ß (0.1ng/ml) for 48 h according to previous investigations [22]. Cell supernatants were stored and applied to Proteome Profiler™ human chemokine and angiogenesis array kits (R&D Systems, Minneapolis, MN, USA) in order to identify the molecular changes caused by the Sdc-1 knock-down in human endometrial stromal cells (n=4 each). Assays were performed according to manufacturers` instructions with 1 ml cell-culture supernatant. Dot blots were photographed and analyzed with an Alpha Imager camera (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and the Alpha Ease FT 6.0.0. program (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

To investigate the statistical significance of the protein expressions, the Student´s t-test with $p<0.05$, $p<0.02$ and $p<0.01$ and one way post-hoc ANOVA test with $p<0.05$ as cut-offs for statistical significance were carried out.

Results

Immunocytochemistry

The nuclear transfection of Tet(R) - coding plasmid pcDNA6/TR® was proven and evaluated via intensity rating of immunocytochemistry staining by two independent investigators (Figure 1). All six

clones resulting from blasticidin selection were tested and the clone showing the strongest staining intensity for Tet(R) was selected for further transfection with Sdc1 shRNA.

Tet-induction of Sdc-1 knock- down

The induction of Sdc1-RNAi in KdS1 with different tetracycline concentrations (0-1000ng/ml) was tested for 24 and 48h and Sdc1-mRNA was measured quantitatively by RT-PCR (data not shown). A 10^6-fold reduction of Sdc1-mRNA was detected after incubation with 1000ng/ml Tet for 48h compared to untreated KdS1 (data not shown). This concentration was used in the following experiments.

Decidualization

KdS1 cells were incubated with Tet for 48h prior to 72h of decidualization protocol and tested for the expression of common decidualization markers IFGBP1 and PRL. dSt-T1 served as controls. The mRNA expression of PRL and IGFBP-1 showed no differences in dKdS1 versus dSt-T1 (data not shown).

Dot Blots after decidualization

Dot blot arrays for cytokines (Figure 2 A, B, Table 3) and angiogenic factors (Figure 2 C, D, Table 4) were performed after 72h decidualization of dKdS1 vs. dSt-T1 which served as controls.

The chemokines CXCL1, CXCL8, IL-6 and macrophage migration inhibitory factor (MIF) were secreted at significantly higher levels in dKdS1. Comparable amounts of IL-13 were secreted from dKdS1 and dSt-T1.

The expression of several cytokines was restricted only to dKdS1. Secretion of CCL2, CCL5, CXCL10, CXCL11 and soluble intercellular adhesion molecule (sICAM-1) occurred in dKdS1 supernatant only. Furthermore, we detected the peptide hormone granulocyte colony-stimulating factor (G-CSF) and the glycoprotein granulocyte macrophage colony-stimulating factor (GM-CSF), both involved in immune cell differentiation and infection mechanisms, only in dKdS1 (Figure 2 B).

When investigating angiogenic factors after decidualization, dKdS1 supernatant contained significantly more hepatocyte growth factor (HGF, scatter factor), a paracrine factor which stimulates mitogenesis, angiogenesis and tumorigenesis, and pentraxin 3 (PTX3), a soluble molecule which belongs to the innate immune system. On the other hand, secretion of tissue inhibitor of metalloproteinases 1 (TIMP-1) and thrombospondin 1 (TSP1), both components of the extracellular matrix, was significantly increased in dSt-T1.

The secretion levels of the angiogenic factors angiopoietin-1 (Ang-1), angiogenin, IGFBP-2 and -3, matrix metalloproteinase 9 (MMP 9) and urokinase-type plasminogen activator (uPA) were similar in dKdS1 versus controls. CXCL16 could be identified to be exclusively expressed in dKdS1 (Figure 2 D). Results are shown in Table 3 and 4.

Dot Blots after decidualization and IL-1β incubation

Furthermore, dot blot arrays for cytokines (Figure 3 A, B, Table 3) and angiogenic factors (Figure 3 C, D, Table 4) were performed after 72h decidualization followed by 48h of coincubation with IL-1β imitating early embryonic contact in dKdS1 vs. the control dSt-T1.

Secretion of the cytokine MIF was significantly increased in dKdS1, whereas CXCL-8 secretion was higher in dSt-T1 cell culture supernatant. The secretion levels of CXCL1 and IL-6 were similar in dKdS1 and dSt-T1 after IL-1ß stimulation. Several cytokines associated with inflammatory mechanisms were only secreted in dKdS1 supernatant, namely G-CSF, GM-CSF, sICAM-1 and the chemokines CCL2, CCL5, CXCL10 and CXCL11. IL-13 was secreted in dSt-T1 exclusively (Figure 3 B).

Obvious changes also occurred regarding the expression profile of angiogenic factors after embryo imitating IL-1ß contact. HGF and IGFBP-2 were secreted significantly higher in dKdS1 compared to dSt-T1. IGFBP-3, TIMP-1 and TSP1 dominated in dSt-T1 supernatant vs. dKdS1. There were no statistically significant differences for MMP9, PTX3 and uPA in dKdS1 vs. dSt-T1. Ang-1 was exclusively secreted from dSt-T1 and angiogenin and CXCL16 were restricted to dKdS1 (Figure 3 D). Results are shown in Table 3 and 4.

Discussion

We have demonstrated an increase in chemokine and angiogenic factor secretion upon Sdc-1 knock-down in decidualized human endometrial stroma cells. These results are consistent with the role of Sdc-1 as a co-receptor and storage factor for these molecules allowing new insights in interactions and regulations between chemokines and angiogenic factors contributing to an improved understanding of intermolecular network at the embryo-maternal interface. Removal of Sdc-1 from endometrial stroma cell surfaces by the process of ectodomain shedding or down regulation of its expression leads to a down regulation of binding sites for chemokines and angiogenic factors. As discussed in the following paragraphs, the nuclear factor-kappaB (NFκB) signalling cascade seems to be involved in Sdc-1 mediated processes in the human endometrium.

Recent studies suggested that Sdcs and especially Sdc-1 and-4 are involved in modifying the actions of chemokines and angiogenic factors by elongation of the ligand-receptor interactions, storage, establishment of a chemokine concentration gradient, shedding and regulation of growth-factor signalling cascades, respectively [9, 23]. The recent observation that newborn Sdc-1 deficient mice are systemically smaller than their wild-type littermates suggests an important role for Sdc-1 during embryonic development [24]. Since animal models can only provide hints towards the human implantation period, human *in-vitro* cell-culture models may be more suitable to mimic the *in-vivo* situation of the human species. The immortalized, non-cancerogenous human endometrial stroma cell line St-T1 was demonstrated to function as an excellent model for human endometrium before [13, 14]. Therefore, this model was used in this study.

The precise molecular actions of Sdc-1 underlying the morphological and structural changes during the menstrual cycle in preparation for embryo implantation or menstrual shedding in absence of fertilization are unknown until today. This study provides the first evidence of an important contribution of Sdc-1 in regulating chemokine and growth factor action during the decidualization and implantation processes.

Selected findings are discussed further in the following paragraphs.

Chemokines in decidualization and early embryo-maternal dialogue

The glutamic acid-leucine-arginine (ELR) motif-positive chemokines CXCL1 and CXCL8 were secreted at higher levels in the Sdc-1 knock-down cells following decidualization, as compared to controls. Regarding secretion after contact with the embryo surrogate, IL-1ß, CXCL1 expression was comparable in both groups, whereas CXCL8 increased significantly in dSt-T1 supernatant compared to dKdS1. ELR-positive CXC-chemokines display angiogenic abilities, whereas chemokines lacking the ELR-motif are often characterized as angiostatic factors [25]. CXCL1 and CXCL8 bind to CXC-receptor 1 (CXCR1) and CXCR2, respectively [26, 27]. Sdc-1 was shown to interact with CXCL1, CXCL8 and their receptors via its anionic heparan sulfate chain [28, 29]. As Sdc-1 might be a storage molecule for chemokines, and has been shown to establish functional gradients for CXCL1 and CXCL8, the increase in CXCL1 and CXCL8 secretion in dKdS1 compared to normal dSt-T1 upon decidualization is most likely due to the absence of Sdc-1 in dKdS1 cells [28, 29]. Furthermore, the rise of CXCL8 after embryo contact in normal decidualized cells links this molecule to early angiogenesis. Interestingly, CXCL8 remains low in dKdS1 after incubation with IL-1ß and nearly equal amounts of CXCL1 are secreted in dKdS1 and dSt-T1. This might be a hint for further chemokine intermolecular networks or a possible function of Sdc-1 in regulating the CXCL1 and CXCL8 signalling. Recent studies focused on Sdc-1´s role in promoting tumour invasion in endometrial cancer cell lines *in-vitro* via NF-κB signalling [30]. This signalling cascade was also shown to be involved in CXCL1 signalling in esophageal cancer and in CXCL8 secretion in primary human hepatocytes [31, 32]. The reduced CXCL8 secretion in dKdS1 cells after IL-1ß contact might be based on a lack of NF-κB signalling or limited CXCL1 and CXCL8 synthesis in dKdS1.

C-C motif chemokine ligand 2 (CCL2, formerly monocyte chemotactic protein-1, MCP-1) was described to be secreted into the uterine lumen as well as in the endometrial stroma by human primary endometrial epithelial cells and in first trimester decidua tissue functioning as a key player of monocyte chemotaxis [33, 34]. Furthermore, CCL2 and its receptor CCR2 were detected in human first trimester decidual tissues with CCL2 being constantly secreted by decidual stromal cells via extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) signalling [35]. Since we detected CCL2 only in dKdS1 supernatant upon decidualization and imitation of embryo contact, we propose an active role for Sdc-1 in binding of CCL2 in human decidualized

endometrium *in vitro*. This storage of CCL2 by Sdc-1 in dES *in vitro* seems to be inconsistent with the *in vivo* situation of early pregnancy described in the literature [35]. Hence, there might be a temporal regulation of CCL2 expression as these data reflect the very early embryo-maternal dialogue. The absence of CCL2 expression in dES *in vitro* might result from the absence of factors that influence the decidua *in vivo*.

Two further CXC-motif chemokines - CXCL10 and 11 - were only secreted in dKdS1 with and without IL-1ß coincubation. The secretion of CXCL10 and 11 might result from the lack of Sdc-1 in dKdS1 cell membranes. In dSt-T1, these molecules are supposingly stored at the cell membrane via Sdc-1 inhibiting early secretion in the supernatant. Therefore, the secretion of these factors in the environment might lead to a shift in the early immune response of the endometrium and a misdirected angiogenesis, respectively.

Another chemokine, macrophage migration inhibitory factor (MIF), was significantly more secreted in dKdS1 after decidualization as well as after incubation with IL-1ß compared to controls. MIF binds to G-protein coupled receptors CXCR2 and CXCR4 and is involved in monocyte and T-cell chemotaxis as well as in activation of integrins and calcium influx [36]. In human endometrium, an increase in MIF expression was found in the late proliferative phase followed by a decline during the window of implantation suggesting different roles for MIF during the menstrual cycle [37]. In our opinion, MIF takes part in the endometrial decidualization process supported by the presence of MIF in decidualized endometrial cells (dSt-T1) where its secretion seems to be regulated by Sdc-1. This regulation might also be initiated by NF-κB signalling similar to the findings in human primary endometrial cells [38].

Angiogenic and growth factors in decidualization and early embryo-maternal dialogue

The process of endometrial angiogenesis already starts in the proliferative phase of the human menstrual cycle and peaks in the secretory phase. A synchronized pattern of angiogenic factors, growth factors and their inhibitors is therefore a critical precondition supporting not only angiogenesis but also modification of the extracellular matrix, decidualization and re-organization of the endometrial stroma.

The paracrine and angiogenic active, glycosaminoglycan-binding hepatocyte growth factor (HGF) was characterized as an essential key player of trophoblast invasion mediating trophoblast growth and differentiation via the signal transducers and activators of transcription (STAT3) signalling pathway via its cytotrophoblast localized receptor c-mesenchymal-epithelial transition factor (c-Met) [39-41]. Furthermore, HGF takes part in embryonic mesenchymal-endothelial interactions and organogenesis as well as in endometrial stromal cell invasion [42, 43]. Sdc-1 has been assigned as a HGF co-receptor intensifying its signalling via c-Met [44]. The significant increase of HGF in dKdS1 supernatant after decidualization and after IL-1ß contact as seen in this study, suggests an intense interaction between Sdc-1 and HGF. It seems that Sdc-1 is important in HGF-binding and presentation towards its receptor for supporting cell proliferation and angiogenesis during the implantation period.

Furthermore, we were able to show that the extracellular matrix glycoprotein thrombospondin 1 (TSP1) was significantly more secreted by normal decidualized and IL-1β incubated dSt-T1 compared to dKdS1. Previous studies focussed on its involvement in endometrial vascularization and decidualization mediated by interferon γ (IFNγ). A modified maternal immune response due to a reduced TSP1 expression of decidual macrophages was described in women with unexplained recurrent embryonic miscarriage [45]. Sdc-1 was found to interact with TSP1 in the formation of stable cellular matrix contacts via fascin spikes [46]. We suggest that the lack of TSP1 secretion in dKdS1 is based on the absence of the known NF-κB activation by IL-1β in dKdS1. Therefore, the absence of TSP1 might lead to angiogenic malformation and dysregulation of the decidualized endometrium possibly leading to implantation failure.

The progesterone-dominated secretory phase endometrium undergoes dramatic changes in matrix reconstruction and differentiation depending on an orchestrated pattern of proteases and inhibitors. Matrix metalloproteinases (MMPs) play an important role during the implantation phase regulated by their inhibitors, e.g. tissue inhibitor of metalloproteinase 1 (TIMP1), localized in fetal and maternal tissues [47, 48]. TIMP1 is the most secreted factor in dSt-T1 after decidualization and IL-1β stimulation. The knock-down of Sdc-1 in dKdS1 might therefore lead to a dysregulation of the TIMP-1 expression. Former studies showed an involvement of Sdc-1 in MMP-9 regulation, a main target of TIMP-1, mediating endometrial cancer invasion [30]. Furthermore, decreased secretion of TIMP-1 in

MCF-7 breast cancer cells overexpressing soluble Sdc-1 ectodomain, supporting breast cancer invasiveness, was described [49]. A decreased expression of TIMP-1 might result in a dysregulated implantation as similar levels of MMP-9 were shown in this study.

The secretion of long pentraxin 3 (PTX3, formerly called pentraxin-related gene rapidly induced by IL-1β) was most significantly elevated in dKdS1 supernatant upon decidualization. PTX 3 is a factor of the innate immune system and is expressed by a huge variety of cells, including macrophages, endothelial cells, fibroblasts and monocytes [50]. Interestingly, PTX 3 was detected in human and mouse cumulus-oocyte complex and $PTX3^{-/-}$ mice show a lack of fertilization in-vivo, but not in-vitro [51]. Recent studies report a distinct role for PTX3 mediating decidualization and fertilization in mice [52]. The increase in PTX3 in dKdS1 supernatant upon decidualization might result from a stronger inflammatory response in dKdS1 as indicated by the elevated secretion levels of other chemokines, e.g. CXCL8, compared to dSt-T1. Moreover, a possible dysregulation of components of the extracellular matrix caused by the Sdc-1 knock-down might activate the PTX3 release in order to induce apoptosis, as it was reported for human neutrophils [53].

Infection-associated molecules in decidualization and early embryo-maternal dialogue

The modulation of the maternal immune system enabling an embryonic invasion is one of the key processes of the early fetal-maternal communication. The important role of cytokines, like IL-1ß system, influencing decidualization- and implantation-related molecules, like MMPs, has already been reported [54]. A recent study examined the components of endometrial secretions aspirated prior to embryo transfer in IVF and ICSI cycles, revealing the presence of pro-, as well as anti-inflammatory immuno-associated molecules [55]. The pro-inflammatory cytokine IL-6 belonged to the main secretion products. In the present study, IL-6 secretion significantly increased in dKdS1 after decidualization. The increase in IL-6 secretion might be due to the lack of Sdc-1 and displays a potential intermolecular interaction between IL-6 and Sdc-1. These data correlate well with observations in Sdc-1 deficient mice subjected to kidney and allergic inflammation where an increase of IL-6 was observed [56]. Hence, the lack of Sdc-1 might induce an inflammatory response in dKdS1

upon decidualization. The similar expression of IL-6 in both groups upon IL-1ß incubation might result from a balancing effect of other factors that are activated upon IL-1ß contact in dKdS1.

The cytokines granulocyte (G-) and granulocyte-macrophage (GM-) colony stimulating factors (CSF) are involved in the implantation period and early fetal maternal dialogue. The interaction between G-CSF and heparan sulfate has been proven in human long-term culture-initiating cells being raised upon a stromal feeder layer [57]. Herein, we detected G-CSF as well as GM-CSF secretion only in dKdS1 with an increase after IL-1ß incubation which underscores a very likely interaction of these colony-stimulating factors with Sdc-1.

Conclusions

The present study therefore underscores the importance of Sdc-1 as a co-receptor and a storage molecule in receptive endometrium supporting a proper biochemical foundation for embryonic implantation. Further investigation of Sdc-1s role in the process of implantation is needed, since its synchronous regulation of multiple receptor-dependent pathways seems to be a pivotal point in understanding and possibly ameliorating human implantation.

List of abbreviations

Ang - angiopoietin, CCL/CXCL - chemokine ligand, c-Met/HGFR - c-mesenchymal-epithelial transition factor/HGF receptor, CXCR - chemokine ligand receptor, d - decidualized, ELR - glutamic acid-leucine-arginine, ERK - extracellular signal-regulated kinase, G-/GM-CSF - granulocyte-/granulocyte-macrophage colony stimulating factor, HGF - hepatocyte growth factor, IFN - interferon, IGFBP - insulin-like growth factor binding protein, IL - interleukin, KdS1 - endometrial Sdc-1 knock-down cell line, LH - luteinizing hormone, MAPK - mitogen-activated protein kinase, MIF - macrophage migration inhibitory factor, MMP - matrix metalloproteinase, NF-κB - nuclear factor kappa B, PTX3 - pentraxin 3, Sdc - Syndecan, shRNA - short hairpin RNA, sICAM - soluble

intercellular adhesion molecule, St-T1 - immortalized endometrial stroma cell line, VEGF - vascular endothelial growth factor, uNK - uterine natural killer cells, Tet - tetracycline, TIMP - tissue inhibitor of metalloproteinases, TSP - thrombospondin, uPA - urokinase-type plasminogen activator

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DMBB Experimental outline, design of short hairpin RNAs, cell culture and decidualization, primer design for RT-PCR, establishment of knock down cell line KdS1, RNA Isolation, Dot Blot analysis, interpretation of the data, manuscript writing

MG Knowledge transfer regarding Syndecan-1, *real-time* PCR, interpretation of the data

WJ Editing of manuscript

JSK Statistical analysis of data, editing of manuscript

APH Idea of the study, experimental outline, immunocytochemistry, interpretation of the data, manuscript writing

All authors read and approved the manuscript.

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Figures

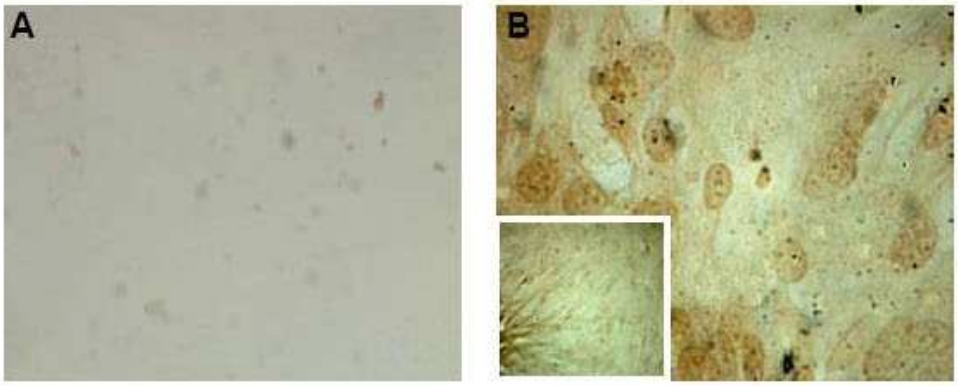

Figure 1 - Nuclear transfection of TetR in St-T1

Representative immunocytochemic staining for nuclear TetR-expression in pcDNA6/TR© transfected St-T1 (1000× magnification, inserts 400×) showing the clone with the most intense staining, A) negative control with non-specific IgG, B) the generated clone.

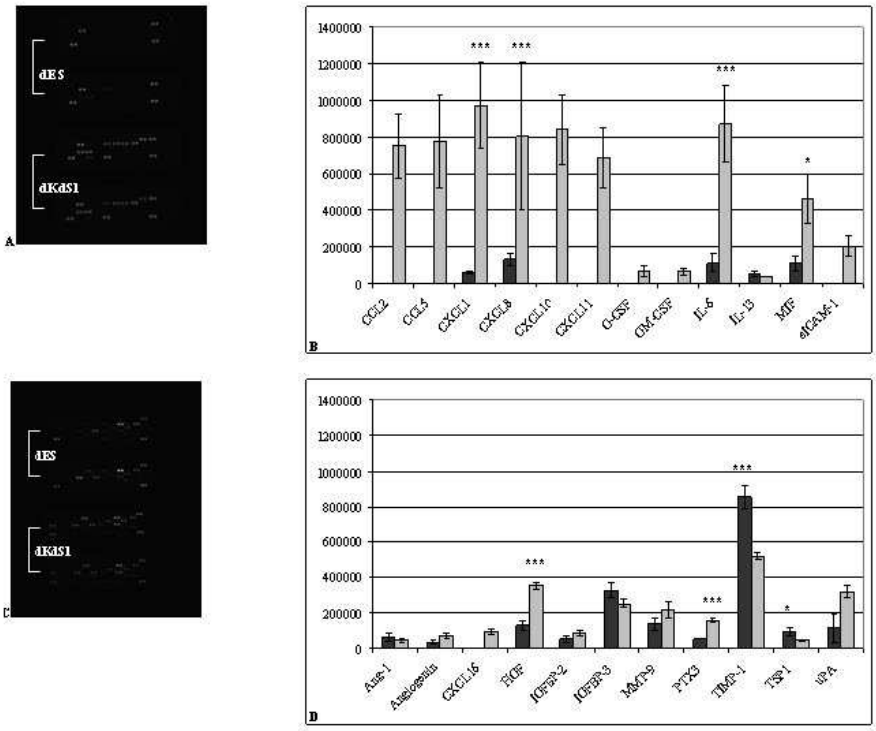

Figure 2 - Cytokine and angiogenic factor profiling after decidualization

Pixel density analysis of cytokine (A, B) and angiogenic factor (C, D) dot blot array of decidualized dSt-T1 (dark grey bars) and dKdS1 (light grey bars) (n=4 each) (*p<0.05, **p<0.02, ***p<0.01). A, C) representative dot blots of cell-culture supernatant, B) corresponding pixel analysis of secreted cytokines, D) corresponding pixel analysis of secreted angiogenic factors.

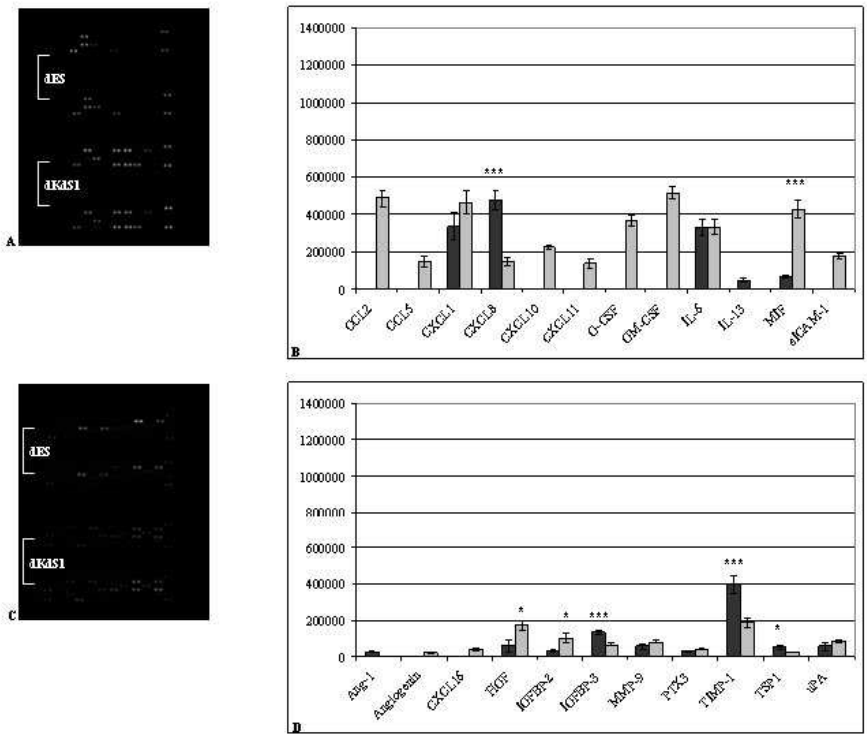

Figure 3 - Cytokine and angiogenic factor profiling after IL-1ß contact

Pixel density analysis of cytokine (A, B) and angiogenic factor (C, D) dot blot array of decidualized dSt-T1 and dKdS1 followed by an IL-1ß incubation for 48h (n=4 each) (*p<0.05, **p<0.02, ***p<0.01). A, C) representative dot blots of cell-culture supernatant, B) corresponding pixel analysis of secreted cytokines, D) corresponding pixel analysis of secreted angiogenic factors.

Tables

No.	sequence shRNA (5´➔3´) (*loop sequence*)	database sequence NM_002997
1	**top** cac cag gac ttc acc ttt gaa acc *cga agg* ttt caa agg tga agt cc **bottom** aaa agg act tca cct ttg aaa cc*t tcg* ggt ttc aaa ggt gaa gtc ct	^{874}agg act tca cct ttg aaa cc^{893}
2	**top** cac cag gag gaa ttc tat gcc tga *cga a*tc agg cat aga att cct cc **bottom** aaa agg agg aat tct atg cct ga*t tcg* tca ggc ata gaa ttc ctc ct	^{1162}agg agg aat tct atg cct ga^{1181}
3	**top** cac cgg taa gtt aag taa gtt ga*c gaa* tca act tac tta act tac c **bottom** aaa agg taa gtt aag taa gtt ga*t tcg* tca act tac tta act tac c	^{1749}ggt aag tta agt aag ttg a^{1767}

Table 1 - Design of Sdc-1 shRNAs

Sequences of shRNAs versus Sdc-1 with siRNA sequences underlined

	sequences 5´➔ 3´	size of the amplified fragment [bp]
β-actin	**for** - cagggtgtgatggtgggaatgg **rev** - caggatggcgtgagggagagca	409
IGFBP-1	**for** - agtttagccaaggcacagga **rev** - tatctggcagttggggtctc	204
PRL	**for** - gcttctgtatcatctggtcacg **rev** - tgcgtaggcagtggagcag	247
GAPDH	**for** - tgcaccaactgcttagc **rev** - acagtcttctgggtggcagtg	131

Table 2 - PCR and RT-PCR primers

Primers for amplification with PCR and RT-PCR

cytokines	dSt-T1	dKdS1	dSt-T1+IL-1β	dKdS1+IL-1β
CCL2		+		+
CCL5		+		+
CXCL1	+	+	+	+
CXCL8	+	+	+	+
CXCL10		+		+
CXCL11		+		+
G-CSF		+		+
GM-CSF		+		+
IL-6	+	+	+	+
IL-13	+	+	+	
MIF	+	+	+	+
sICAM-1		+		+

Table 3 - Cytokine profiling

Overview of cytokines in cell culture supernatant of decidualized endometrial stroma cells (dSt-T1), decidualized Sdc-1 knock down endometrial stroma cells (dKdS1), decidualized endometrial stroma cells coincubated with IL-1β (dSt-T1 + IL-1ß) and decidualized Sdc-1 knock down endometrial stroma cells coincubated with IL-1β (dKdS1 + IL-1ß). + indicates the presence, - the absence of the respective cytokine in the cell culture supernatant.

angiogenic factors	dSt-T1	dKdS1	dSt-T1+IL-1β	dKdS1+IL-1β
Ang-1	+	+	+	
Angiogenin	+	+		+
CXCL16		+		+
HGF	+	+	+	+
IGFBP-2	+	+	+	+
IGFBP-3	+	+	+	+
MMP-9	+	+	+	+
PTX3	+	+	+	+
TIMP-1	+	+	+	+
TSP-1	+	+	+	+
uPA	+	+	+	+

Table 4 - Angiogenic factor profiling

Overview of angiogenic factors in cell culture supernatant of decidualized endometrial stroma cells (dSt-T1), decidualized Sdc-1 knock down endometrial stroma cells (dKdS1), decidualized endometrial stroma cells coincubated with IL-1β (dSt-T1 + IL-1ß) and decidualized Sdc-1 knock down endometrial stroma cells coincubated with IL-1β (dKdS1 + IL-1ß). + indicates the presence, - the absence of the respective angiogenic factor in the cell culture supernatant.

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2.5 *In-vitro* culture does not alter the expression of vascular endothelial growth factor and its receptors in single murine preimplantation embryos (accepted for publication in Gynecologic and obstetric investigation)

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Vasculogenesis, the de novo formation of the embryonic vasculature, and angiogenesis, the growth of blood vessels from pre-existing ones, are essential for the establishment of a successful pregnancy in many species. The human VEGF family consists of 5 different members (VEGF-A, -B, -C and –D and placental growth factor (PlGF) all of them sharing eight highly conserved cysteine residues. Gene knock-out studies in mice emphasized the importance of VEGF and its receptors VEGFR1 (formerly known as Flt-1) and VEGFR2 (formerly known as KDR). The loss of a single VEGF allele alone leads to severe developmental deformities in the growing vasculature and embryonic death between days 11 and 12. Both membranous receptors VEGFR1 and VEGFR2 have seven immunoglobulin-like extracellular domains, a transmembranous region and a cytoplasmic tyrosine-kinase sequence with 33 % identity of the amino acid sequence. Binding of the ligand leads to dimerization, autophosphorylation and phosphorylation of other activators involved in signal transduction (e. g. GTPase activating enzyme). The transcription of VEGF and its receptors is described in several tissues of the female reproductive tract, e. g. corpora lutea, endometrium, follicles and placenta as well as at the implantation site in the mouse-model. The complex process of embryonic implantation is characterized by a highly orchestrated feto-maternal communication, including processes as angiogenesis, matrix degeneration and modulation of the immune system. Therefore VEGF and its receptors seem to play an important role in the early establishment of pregnancy. Regarding assisted reproduction techniques (ART), scientists try to mimic the female reproductive tract for *in vitro* fertilization and embryo culture by optimizing culture media for decades now. Studies employing mouse and human embryos to investigate different culture media and their impact on embryo morphology, cleavage rates, blastocyst development and implantation rate revealed a

considerable impact of the media and respectively of the resulting pregnancy rates. Furthermore, since the introduction of intracytoplasmic sperm injection technique (ICSI) in ART the overall pregnancy rate in *in vitro* fertilization (IVF) cycles remained almost invariant within the last years indicating that yet another advancement in pregnancy rate might only be achieved by improving the embryo culture condition.

The aim of this study was to detect possible alterations of VEGF and its receptors mRNA expression in murine embryos in their development from 8-cell stage to late blastocyst and furthermore a possible impact of *in vitro* culture compared to *in vivo* development.

A total number of 139 single blastocyst (77 *in vivo* blastocyst and 62 *in vitro* blastocyst) were examined for β-actin-, VEGF-, VEGFR1- and VEGFR2- mRNA-expression.

All blastocyst – cultured *in vivo* and *in vitro* - were positive for β-actin mRNA-expression. 87% of the blastocyst of the *in vivo* section expressed VEGF mRNA almost comparable to the *in vitro* group (81%). The VEGFR1 mRNA was expressed in 2.6% of the *in vivo* and in 3.2% of the *in vitro* cultured embryos. VEGFR2 was detected in 6.5 % of the examined blastocyst in both groups. All embryos expressing mRNAs for the receptors were positive for VEGF, too.

There was no significant difference between the mRNA expression profile examined (confirmed by a correlation coefficient of 0.99) of all *in vivo* developed versus *in vitro* cultured blastocyst.

Concerning the developmental expression of the VEGF system members *in vitro*, 43 8-cell-stage, 51 morula, 43 early and 36 late blastocyst were examined. 4, 7, 5 and 3 preimplantation embryos, respectively, were excluded due to a lack of β-actin expression. We could detect a higher number of embryos with positive VEGF-mRNA expression according to the developmental stage. Except for the 8 cell –and morula stage embryos, VEGFR1-mRNA was expressed in 50% of early and late blastocyst. Nearly 70% of 8-cell stage and early blastocyst expressed VEGFR2-mRNA in contrast to nearly 20% of morula and late blastocyst.

Implantation of a blastocyst into a receptive endometrium is a necessary prerequisite for successful pregnancy. High levels of VEGF are generally associated with hypoxia. Many cytokines and growth factors as TNF-α, TGF-β, epidermal growth factor (EGF), IL-1β , IL-6 and insulin-like growth factor 1 (IGF-1) up regulate VEGF mRNA or induce VEGF release. Some of them lack direct angiogenic effects but exert angiogenic activity through VEGF.

Hypoxia induces a rapid and strong increase in VEGF mRNA levels *in vivo*, which makes it a target for changes due to *in vitro* culture with higher oxygen levels.

Especially the culture medium, fluctuations in pH and temperature, exposure to light outside the incubator and elevated oxygen tension within the culture period are of concern. Over the last decades researchers tried to optimize the *in vitro* environment by co-culture strategies involving human heterologous as well as autologous oviductal or endometrial epithelial cells mimicking the *in vivo* situation. Also a lot of studies focused on the development of more suitable media for embryo culture. Additionally embryos are generally transferred into the uterus one to two days before the normal entry time into the uterus given a standard culture time of 48 to 72 hours *in vitro*. Since this study was carried out in the mouse model for ethical reasons and therefore a system was applied that differs in physiology and implantation mechanism compared to the human model possible variations of the described data might occur. Nevertheless the similarity in metabolic parameters between mouse and human embryos has facilitated the use of the mouse model to study culture media to improve the outcome of human IVF technique in the past decades so that we consider it an appropriate substitute in our study.

This is the first report comparing the expression of VEGF and its receptors VEGFR1 and VEGFR2 of murine preimplantation blastocyst left *in vivo* versus blastocyst cultured for 3 days under *in vitro* conditions showing no changes due to the artificial *in vitro* culture outside the maternal uterus regarding the investigated growth factor and its receptors. These findings are reassuring for the safety of traditional assisted reproduction techniques since VEGF is one of the major key players in terms of angiogenesis induction which is of extreme importance for a successful establishment of pregnancy.

The presence of the growth factor VEGF and its receptors underlines the interaction between preimplantation embryo and decidualized endometrium supporting vasculogenesis and angiogenesis at the implantation site and within the embryo.

Declaration of DM Baston-Büst´s role:

interpretation of the data, writing of the manuscript

In-vitro culture does not alter the expression of vascular endothelial growth factor and its receptors in single murine preimplantation embryos

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Keywords: angiogenesis, mouse preimplantation embryo, VEGF, VEGFR1, VEGFR2

Abstract

Background: *In vitro* culture of embryos, as widely used in assisted reproduction techniques, may influence embryonic development and subsequently the establishment of pregnancy.

The aim of this study was to determine a potential influence of the *in vitro* culture regarding VEGF, VEGFR1 and VEGFR2 mRNA expression in developing single mouse embryos.

Methods: Murine embryos were isolated on day 1 post coital (pc) and cultivated for a developmental time course followed by examination for mRNA expression using RT-nested PCR. Furthermore, *in vitro* cultured blastocysts were compared to *in vivo* development at 101 hours pc.

Results: At 101 hours pc there were no significant differences between in vivo and in vitro cultured blastocysts regarding the expression of VEGF and its receptors. In the developmental time course, VEGF expression increased up to 94 % in late blastocysts whereas the VEGF receptor expression remained low.

Conclusions: This study showed that the *in vitro* culture did not alter the embryonic VEGF and VEGFR mRNA expression reassuring that the culture conditions in assisted reproduction techniques are well suited for maintaining the VEGF mRNA expression profile. Additionally nearly 100 % VEGF expression in late blastocysts highlights its importance for angiogenesis induction at the fetal-maternal interface.

Introduction

Vasculogenesis, the de novo formation of the embryonic vasculature, and angiogenesis, the growth of blood vessels from pre-existing ones, are essential for the establishment of a successful pregnancy in many species [1]. The angiogenic growth-factor VEGF (vascular endothelial growth factor) has first been characterized in vascular endothelial cells of the bovine brain [2,3]. The human VEGF family consists of 5 different members (VEGF-A, -B, -C and –D and placental growth factor (PlGF) all of them sharing eight highly conserved cysteine residues. Gene knock-out studies in mice emphasized the importance of VEGF and its receptors VEGFR1 (formerly known as Flt-1) [4] and VEGFR2 (formerly known as KDR) [5]. The loss of a single VEGF allele alone leads to severe developmental deformities in the growing vasculature and embryonic death between days 11 and 12 [6,7]. Mice lacking either VEGFR-1 or VEGFR-2 die even earlier, between embryonic day 8.5 and 9.5 [8,9]. In the $VEGFR1^{-/-}$ mice, endothelial cells form but fail to organize properly into blood vessels which seem to be a consequence of increased commitment of mesenchymal cells becoming hemangioblasts, the common precursors of both blood cells and endothelial cells [10]. Whereas $VEGFR2^{-/-}$ mice display defects in the development of endothelial and hematopoietic precursor cells [9].

Both membranous receptors VEGFR1 and VEGFR2 have seven immunoglobulin-like extracellular domains, a transmembranous region and a cytoplasmic tyrosine-kinase sequence with 33 % identity of the amino acid sequence [11]. Binding of the ligand leads to dimerization, autophosphorylation and phosphorylation of other activators involved in signal transduction (e. g. GTPase activating enzyme) [5,12]. The transcription of VEGF and its receptors is described in several tissues of the female reproductive tract, e. g. corpora lutea, endometrium, follicles and placenta as well as at the implantation site in the mouse-model [13-15]. Vasculogenesis and subsequent angiogenesis are key events in this process but as of

today the mechanisms by which localized changes in vasculogenesis, vascular permeability and angiogenesis occur are not fully understood. VEGF and its receptors VEGFR-1 and -2 have been implicated as important factors in vascular remodelling and placentation though. VEGF receptor expression was thought to be exclusive to vascular endothelial cells but in addition binding of VEGF to non-endothelial cells was found suggesting an alternative function such as induction of monocyte migration [16]. It was shown that VEGF application resulted in an increased cell survival of dopaminergic cells and axone growth in a rat model of Parkinson`s disease [17]. Furthermore, VEGF is involved in repair and anti-apoptotic mechanisms in central and peripheral nervous system [18]. Hence, VEGF is a multifunctional molecule involved in different essential mechanisms, e.g. vasculogenesis, angiogenesis, tissue organization and repair.

The complex process of embryonic implantation is characterized by a highly orchestrated feto-maternal communication, including processes as angiogenesis, matrix degeneration and modulation of the immune system [19,20]. Therefore VEGF and its receptors seem to play an important role in the early establishment of pregnancy. Regarding assisted reproduction techniques (ART), scientists try to mimic the female reproductive tract for *in vitro* fertilization and embryo culture by optimizing culture media for decades now. Since the rate of specific pathologic syndromes, e. g. Beckwith-Wiedemann syndrome or large-offspring syndrome known from veterinary embryo *in vitro* culture possibly resulting from imprinting disorders or potentially elongated culture of blastocyst raises the question whether the culture media in human *in vitro* systems may alter the protein expression profile of developing embryos as well and therewith probably introducing a negative implanting capacity or even worse occurrence of syndromes in the off spring. Studies employing mouse and human embryos to investigate different culture media and their impact on embryo morphology, cleavage rates, blastocyst development and implantation rate revealed a considerable impact of the media and respectively of the resulting pregnancy rates [21-24]. Furthermore, since the

introduction of intracytoplasmic sperm injection technique (ICSI) in ART the overall pregnancy rate in *in vitro* fertilization (IVF) cycles remained almost invariant within the last years indicating that yet another advancement in pregnancy rate might only be achieved by improving the embryo culture condition.

The aim of this study was to detect possible alterations of VEGF and its receptors mRNA expression in murine embryos in their development from 8-cell stage to late blastocyst and furthermore a possible impact of *in vitro* culture compared to *in vivo* development.

2. Materials and methods

2.1 Animals

Planning and conduction of the experimental procedures as well as maintenance of the animals was carried out in accordance to the german Guide for the Care and Use of Laboratory animals and the ethics board of the Heinrich Heine University. 25 female, 12 week old mice from the B6C3F1 strain were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA, USA) and maintained at 22-24°C on a 12h light – 12h dark cycle. Female mice were superovulated by intraperitoneal (ip) injection of 10IU pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich, Seelze, Germany). 48h after PMSG, ovulation was induced by ip injection of 10IU human chorionic gonadotropin (hCG, Sigma-Aldrich). Female mice were impregnated by 12 week old fertile males of the same strain. A single male was placed with two females overnight. Mating was verified by the appearance of a vaginal plug on the following morning.

2.2 Zygote recovery and embryo culture

24h after hCG injection, mated mice were sacrified by cervical dislocation. Ovaries and oviduct were removed and washed with universal IVF-medium (Medicult, Jyllinge, Denmark) with 80IU/ml hyaluronaidase (Sigma-Aldrich). Cumulus-zygote-complexes were removed from the oviducts by cutting the swollen parts of the ampulla, gently squeezing the oviduct and flushing with medium under visual control. Cumuli were removed by incubation in 80IU/ml hyaluronidase (Sigma-Aldrich) for 3min and gentle suction through a Pasteur pipette (Fisher Scientific, Schwerte, Germany). Cumulus-free zygotes were transferred to falcon culture dish (Becton Dickinson, Heidelberg, Germany) and cultured at 37°C in a humidified atmosphere of 5% CO_2/ 95% air. The culture dishes were prepared as follows: 25µl G1 medium (IVF Science Colorado, Inc., Englewood, Colorado, USA) were placed in the dish and covered with light weight mineral oil (Sigma-Aldrich). Embryos were observed 42h (2-cell-stage), 67h (8-cell-stage), 88h (compacted morula) and 101h post hCG injection (late blastocyst) to monitor their development and were eliminated in case of an developmental arrest (Fig. 1) and harvested for time course development, respectively. 72h p. hCG injection embryos were transferred into G2 medium (IVF Science Colorado). The corresponding *in vivo* blastocyst for comparison were removed 101h p. hCG injection from the mouse uteri by flushing the uteri with a microneedle containing universal IVF medium (Medicult) under visual control. Then single blastocyst were transferred into thin wall PCR tubes (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) for further experiments. Single embryos were examined for mRNA-levels of β-actin, VEGF, VEGFR1 and VEGFR2 by two rounds of nested polymerase chain reaction (PCR). A total of 74 embryos were investigated in the *in vivo* group and 228 *in vitro*.

2.3 Primers for reverse transcription (RT) and polymerase chain reaction (PCR)

Sequences of cDNA clones for the mRNAs investigated in single mouse embryos β-actin [Accession number M12481; [Alonso S, 1986 #19]], VEGF splice variant VEGF-A (exons 1-5 were amplified with the outer primer pair and exons 3 and 4 with the inner one) [M95200; [Claffey KP, 1992 #20]], VEGFR1 [D88689; [Finnerty H, 1993 #21]] and VEGFR2 [X70842; [Millauer B, 1993 #22]] were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institute of Health (NIH) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide&itool=toolbar). The VEGFR1 primer pair was designed to avoid detection of the soluble antagonistic form of the receptor soluble flt-1. One set of corresponding outer primer-sequences and one set of corresponding inner primer-sequences were constructed using OLIGO 4.1 Primer Analysis Software (National Bioscience, Plymouth, MN, USA) and synthesized by Eurofins MWG Operon (Ebersberg, Germany) (Tab. 1). To ensure that the product detected resulted from amplification of cDNA rather than contaminiting genomic DNA, primers were designed to cross intron-exon boundaries. The primer cDNA sequences for and the sizes of the amplicon are listed in Tab. 1. Although the use of two nested primer-pairs should yield in a high specifity for the amplified cDNA, we additionally confirmed the identity of the amplicons of VEGF, VEGFR1 and VEGFR2 by sequence analysis (data not shown) (biomedical research center of the Heinrich Heine University, Duesseldorf, Germany).

2.4 Reverse transcription (RT) reaction

302 embryos were used for RT. For each embryo, 17.5µl RT-master mix was prepared (5mM $MgCl_2$, 1×RT-buffer, dNTPs each 1mM, 0.25µM oligo $d(T)_{16}$, 0.375µM outer 3´ primer-mix, DEPC-treated dH_2O ad 17.5µl) [all Applied Biosystems, Foster City, CA, USA], added to a thin wall PCR-tube (Biozym Scientific GmbH) with a single embryo in 1µl media, covered with light white PCR mineral oil (Sigma-Aldrich) and kept on ice until RT. For RNA-

extraction samples were heated up to 99°C for 1min in a Biometra T-Gradient (Biometra, Goettingen, Germany) to release the total RNA and denature the proteins as described before [25]. Samples were cooled down to 4°C and 0.5U/µl RNase inhibitor and 2.5U/µl MuLV reverse transcriptase (both Applied Biosystems) were added to each reaction [25]. The protocol for RT was as follows: 42°C for 60min, 99°C for 5min, 4°C for ∞. After the reaction was complete, samples were diluted with DEPC-treated dH_2O ad 50µl and stored at -20°C until the PCR reaction was carried out.

2.4 Nested Polymerase chain reaction (PCR)

A total of 5µl of diluted RT-product was added to 45µl of specific PCR-1-mix for VEGF, VEGFR1 and VEGFR2 (1.9mM $MgCl_2$, 1×PCR-buffer, dNTPs each 1mM, outer primer-pair mix each 0.5µM, 2.5U/µl AmpliTaq® DNA-polymerase, DEPC-treated dH_2O ad 45µl) (all Applied Biosystems) and heated to 95°C for 5min to activate the hot-start enzyme. dH_2O and template omission served as negative controls. After completion of 40 cycles of 94°C for 45sec, 54°C for 45sec and 72°C for 60sec, the reaction was terminated at 72°C for 5min and cooled down to 4°C ∞. First round PCR-products were stored at -20°C until the second PCR. For the second PCR, 5µl of the first round PCR-product was added to 45µl PCR-2-mix (1.9mM $MgCl_2$, 1×PCR-buffer, dNTPs each 1mM, inner primer-pair mix each 0.5µM, 2.5U/µl AmpliTaq® DNA-polymerase, DEPC-treated dH_2O ad 45µl). Program parameters were identical with the first round protocol except annealing temperatures (for VEGF 54°C; 52°C for VEGFR1 and VEGFR2) and numbers of cycles (35 for VEGF and 40 for VEGFR1, VEGFR2). Samples were stored at -20°C until agarose-gel electrophoresis was carried out.

2.5 Agarose-gel electrophoresis

Horizontal 2% agarose-gel electrophoresis was carried out in the presence of ethidium-bromide [0.5µg/ml] (Sigma-Aldrich). A 100bp DNA ladder (GibcoBRL, Eggenstein, Germany) was used to determine the sizes of the amplified fragments. After completion of electrophoresis, the agarose-gel was analyzed with the GelDoc 1000 system (Bio-Rad Laboratories, Hercules, CA, USA).

2.6 Statistical analysis

Embryonic expression of the investigated mRNAs was encoded as: 0, non-detectable, and 1, detectable, for each investigated embryo in statistical analysis. To investigate the statistical correlation of the different mRNA-expressions of day 3 blastocyst developed *in vivo* compared with *in vitro* culture we carried out an analysis of correlation for linear size as this statistical size can possibly link two different examined groups.

3. Results

A total number of 139 single blastocyst (77 *in vivo* blastocyst and 62 *in vitro* blastocyst) were examined for β-actin-, VEGF-, VEGFR1- and VEGFR2- mRNA-expression.

All blastocyst – cultured *in vivo* and *in vitro* - were positive for β-actin mRNA-expression (tab. 2, 3). 87% of the blastocyst of the *in vivo* section expressed VEGF mRNA almost comparable to the *in vitro* group (81%) (tab. 2, 3). The VEGFR1 mRNA was expressed in 2.6% of the *in vivo* and in 3.2% of the *in vitro* cultured embryos (tab. 2, 3). VEGFR2 was detected in 6.5 % of the examined blastocyst in both groups (tab. 2, 3) (fig. 2 represents quantitative mRNA detection for *in vivo* developed blastocyst via PCR).

All embryos expressing mRNAs for the receptors were positive for VEGF, too.

There was no significant difference between the mRNA expression profile examined (confirmed by a correlation coefficient of 0.99) of all *in vivo* developed versus *in vitro* cultured blastocyst.

Concerning the developmental expression of the VEGF system members *in vitro*, 43 8-cell-stage, 51 morula, 43 early and 36 late blastocyst were examined. 4, 7, 5 and 3 preimplantation embryos, respectively, were excluded due to a lack of β-actin expression. We could detect a higher number of embryos with positive VEGF-mRNA expression according to the developmental stage (tab. 4). Except for the 8 cell –and morula stage embryos, VEGFR1-mRNA was expressed in 50% of early and late blastocyst (tab. 4). Nearly 70% of 8-cell stage and early blastocyst expressed VEGFR2-mRNA in contrast to nearly 20% of morula and late blastocyst (tab. 4).

4. Discussion

Implantation of a blastocyst into a receptive endometrium is a necessary prerequisite for successful pregnancy. High levels of VEGF are generally associated with hypoxia. Many cytokines and growth factors as TNF-α, TGF-β, epidermal growth factor (EGF), IL-1β , IL-6 and insulin-like growth factor 1 (IGF-1) up regulate VEGF mRNA or induce VEGF release. Some of them lack direct angiogenic effects but exert angiogenic activity through VEGF [26]. Hypoxia induces a rapid and strong increase in VEGF mRNA levels *in vivo*, which makes it a target for changes due to *in vitro* culture with higher oxygen levels. Interestingly, the other VEGF family members are not induced by hypoxia; therefore VEGF might be considered the main mediator of hypoxia-induced neovascularisation [26,27].

In vitro culture of preimplantation blastocyst as widely used in assisted reproduction technique is of questionable impact for the developing embryo since the culture time is possibly associated with a developmental block or loss of viability [28]. Although no data in

human are available due to ethical reasons animal models clearly show a better embryo development *in vivo* compared to *in vitro*. *In vitro* cultured mouse embryos showed a reduction in the number of trophoblastic cells, the inner cell mass and larger inner cell mass nuclei compared to *in vivo* embryos. [29]. Especially the culture medium, fluctuations in pH and temperature, exposure to light outside the incubator and elevated oxygen tension within the culture period are of concern. Over the last decades researchers tried to optimize the *in vitro* environment by co-culture strategies involving human heterologous as well as autologous oviductal or endometrial epithelial cells [30-32] mimicking the *in vivo* situation. Also a lot of studies focused on the development of more suitable media for embryo culture [21,33,34]. Additionally embryos are generally transferred into the uterus one to two days before the normal entry time into the uterus given a standard culture time of 48 to 72 hours in vitro. Therefore, emerging technologies such as micro fluid systems try to provide a more natural dynamic microenvironment in contrast to the static micro drop culture technology used in the past decades [30-32]. Although these new devices are very promising they need to be tested further with regards to safety and reliability as well as the number of patients treated needs to be enlarged before a superiority compared to the traditional *in vitro* culture system can be established. Hence, the question whether the *in vitro* culture renders the protein expression profile negatively for the developing embryo remains very important.

Since this study was carried out in the mouse model for ethical reasons and therefore a system was applied that differs in physiology and implantation mechanism compared to the human model possible variations of the described data might occur. Nevertheless the similarity in metabolic parameters between mouse and human embryos has facilitated the use of the mouse model to study culture media to improve the outcome of human IVF technique in the past decades so that we consider it an appropriate substitute in our study [34,35].

This is the first report comparing the expression of VEGF and its receptors VEGFR1 and VEGFR2 of murine preimplantation blastocyst left *in vivo* versus blastocyst cultured for 3

days under *in vitro* conditions showing no changes due to the artificial *in vitro* culture outside the maternal uterus regarding the investigated growth factor and its receptors. These findings are reassuring for the safety of traditional assisted reproduction techniques since VEGF is one of the major key players in terms of angiogenesis induction which is of extreme importance for a successful establishment of pregnancy. Nevertheless further investigation of other factors needs to endorse these findings and therewith the safety of *in vitro* culture of human embryos under the given conditions. Over a time course from 8 cell until late blastocyst stage a higher number of embryos expressing VEGF mRNA was shown supporting the theory that VEGF is needed at the embryo-maternal interface for induction of angiogenesis. The presence of the growth factor VEGF and its receptors underlines the interaction between preimplantation embryo and decidualized endometrium supporting vasculogenesis and angiogenesis at the implantation site and within the embryo.

5. Acknowledgements

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Tables

Tab. 1

mRNA	type of oligo-nucleotide	size of the fragment [bp]	5′/3′- end	sequence of oligonucleotide 5′→ 3′
β-actin	one pair	407	5′	CAA GGT GTG ATG GTG GGA ATG G
			3′	CAG GAT GGC GTG AGG GAG AGC A
VEGF	outer pair	365	5′	TGG ACC CTG GCT TTA CTG
			3′	TGG TCT GCA TTC ACA TCT G
	inner pair	188	5′	ATT GAG ACC CTG GTG GAC A
			3′	TGT GCT GGC TTT GGT GAG
VEGFR1	outer pair	775	5′	CAC TGC CAC TCT CAT TGT AA
			3′	ACG AGG TCT CCA TCA GTG G
	inner pair	713	5′	CAA GCC CAC CTC TCT ATC C
			3′	ACG AGG TCT CCA TCA GTG G
VEGFR2	outer pair	672	5′	GCC TCT CAT GGT GAT TGT G
			3′	CCT GAC TAC ACT ACC CCA GA
	inner pair	398	5′	GCA TCA CCA GCA GCC AGA G
			3′	CGG TGT GTT GCT CTG GGA A
Oligo $d(T)_{16}$				TTT TTT TTT TTT TTT T

290 **Tab. 2**

specific mRNA expression	β-actin	VEGF	VEGFR1	VEGFR2
	(total numbers)/%	(total numbers)/%	(total numbers)/%	(total numbers)/%
d3 *in vivo* embryos	(74/74) / 100	(64/74) / 87	(5/74) / 6.5	(2/74) / 2.6

Tab. 3

specific mRNA expression	β-actin	VEGF	VEGFR1	VEGFR2
	(total numbers)/%	(total numbers)/%	(total numbers)/%	(total numbers)/%
d3 *in vitro* cultured embryos	(55/55) / 100	(45/55) / 81	(4/55) / 6.5	(2/55) / 3.2

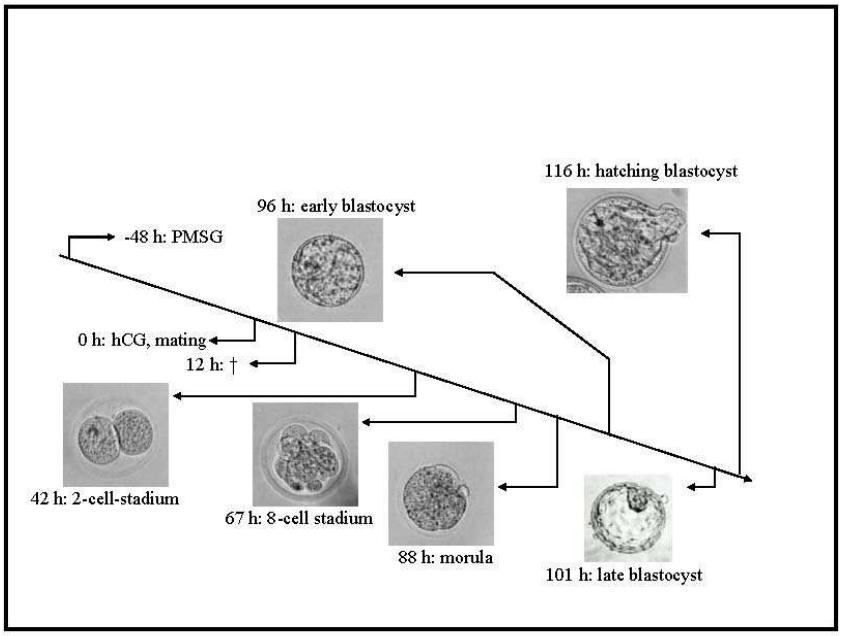
295

Tab. 4

specific mRNA expression of *in vitro* cultured embryos	β-actin	VEGF	VEGFR1	VEGFR2
	(total numbers)/%	(total numbers)/%	(total numbers)/%	(total numbers)/%
8-cell stage	(39/43) / 91	(17/39) / 44	(4/39) / 10	(27/39) / 69
morula	(44/51) / 86	(18/44) / 41	(4/44) / 10	(6/44) / 14
early blastocyst	(38/43) / 88	(23/38) / 61	(19/38) / 50	(25/38) / 66
late blastocyst	(33/36) / 92	(31/33) / 94	(18/33) / 48	(6/33) / 18

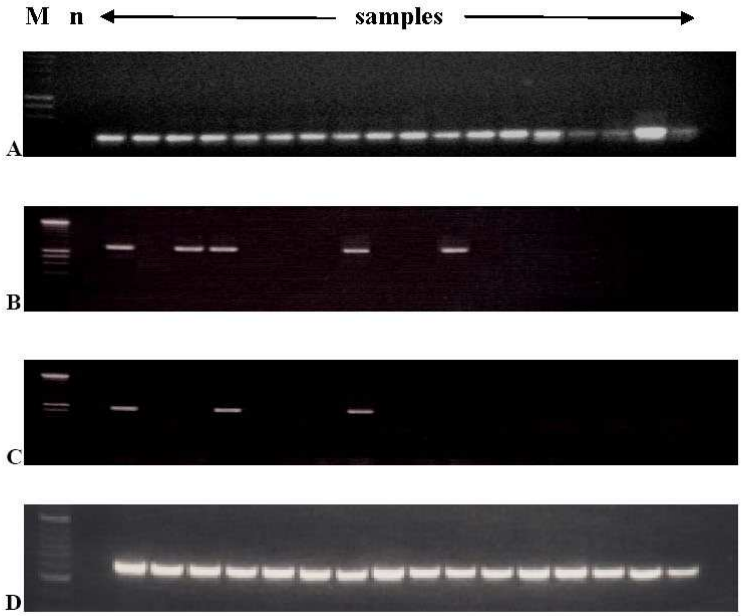
Figures

Fig. 1:



300

Fig. 2:



Legends

Tab. 1

Sequences of oligonucleotides used in reverse transcription and nested PCR.

Tab. 2

RT-PCR quantitative detection of VEGF, VEGFR1 and VEGFR2 (ß-actin served as a housekeeping gene) of day 3 (d3) *in vivo* developed mouse blastocyst. Data are presented as total number in brackets followed by the indication of percentage of each group.

Tab. 3

RT-PCR quantitative detection of VEGF, VEGFR1 and VEGFR2 (ß-actin served as a housekeeping gene) of day 3 (d3) *in vitro* cultured murine blastocyst. Data are presented as total number in brackets followed by the indication of percentage of each group.

Tab. 4

RT-PCR quantitative detection of β-actin, VEGF, VEGFR1 and VEGFR2 in developing murine embryos from 8-cell to late blastocyst stage *in vitro*. β-actin was applied as an internal standard. Data are presented as total number in brackets followed by the indication of percentage of each group.

Fig. 1

Overview representing experimental time course from stimulation of female mice, isolation of embryos and their early development photographed during the *in vitro* culture (modified after Kruessel et al., J Reprod Immunol 1997; 103-120).

Fig. 2

Representative gel electrophoresis of mRNA expression for VEGF (A), VEGFR1 (B) and VEGFR2 (C) and β-actin as internal standard (D) of day 3 murine blastocyst developed *in vivo* representing the embryos´ mRNA expression pattern under physiological conditions
335 (marker for DNA size (M) was followed by negative control (n) and single blastocyst samples (samples)).

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2.6 Expression of the vascular growth factor receptor neuropilin-1 in the human endometrium (Journal of Reproductive Immunology 79 (2009) 129-136) & Erratum (J Reproductive Immunology 81 (2009) 103)

AP Hess, A Schanz, DM Baston-Buest, J Hirchenhain, MA Stoff-Khalili, P Bielfeld, JS Kruessel

The endometrium and its vasculature undergo remarkable histologic and structural changes throughout the female menstrual cycle in preparation for embryonic implantation or subsequent shedding. These changes include tissue growth and remodelling as well as extensive angiogenesis of the pre-existing vessels. Angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for endometrial proliferation and regeneration during the menstrual cycle. It is a process normally only seen in fetal development, wound healing, metastasis and in the female reproductive tract. During the menstrual cycle, expression of VEGF in the endometrium, particular in glandular epithelial and stromal cells, increases in the mid-secretory phase. Furthermore, cell surface receptor cross-linking studies show that VEGF also binds to other receptors than VEGFR-1 and -2 such as neuropilin-1 (NRP-1) a membrane protein expressed in developing neurons regulating nerve fiber guidance. Neuropilins are transmembrane glycoproteins of approximately 140 kDa with an extracellular portion containing three different structural domains, making it unlikely to function as independent receptors. NRP-1 is expressed in endothelial cells binding $VEGF_{165}$ and therewith enhancing the binding of $VEGF_{165}$ to VEGFR2. A study of human and rhesus macaque endometrium showed a high but stable expression of NRP-1 mRNA in vascular endothelium and stromal cells throughout the cycle and an elevated expression during the mid-to late proliferative phase in the luminal epithelium.

The aim of this study was to elucidate the expression pattern of NRP-1 in human proliferative versus secretory phase endometrium.

From a total of 32 endometrial samples, all were positive for β-actin mRNAexpression and therefore considered for further examination. We detected mRNA for NRP-1 in 32 out of 32

(100%) human endometrial samples, covering 22 samples from the proliferative phase of the cycle and 10 samples from the secretory phase of the cycle. Samples from the proliferative phase of the cycle showed a significant higher expression level of NRP-1 mRNA compared to samples from the secretory phase (*t*/*c*-ratio 2.13 vs. 0.84) with a statistical significance of $p = 0.027$. Of the 32 samples investigated for mRNA expression, five samples of the proliferative and five samples of the secretory phase of the cycle were randomly chosen for further immunohistochemical analysis. The slides of the proliferative phase show strong NRP-1 immunoreactivity in the vascular endothelium (Vessel) [+++] as well as in the glandular epithelium (Gland) [+++]. Within the stromal cell compartment, the perivascular area showed the most staining [+++] followed by the remaining stromal compartment with a rather moderate staining [++]. In comparison to the proliferative phase, only a moderate staining [++] in the vascular endothelium followed by a low staining in the stromal compartment [+] and almost no staining [−/+] in the glandular epithelium of secretory phase endometrium occurred.

The regulation of human endometrial angiogenesis is a necessary prerequisite for a temporal and spatial limited change of the tissue construction in accordance to prepare the endometrium for an implanting conceptus or in its absence for a limited shedding. Since it is highly unlikely that one angiogenic factor alone is responsible for these complex changes in the endometrium, our study has addressed the potential regulation and expression of NRP-1, the major known neuropilin isoform facilitating VEGF action, throughout the human menstrual cycle. This study demonstrates mRNA and protein expression of NRP-1 in the human endometrium during the early proliferative and the midsecretory phase of the natural menstrual cycle. The spatial distribution of NRP-1 expression seen in this study resembled the expression pattern of VEGFR-1 and VEGFR-2 found in other studies. The increased NRP-1-expression in the proliferative phase leads to the assumption that NRP-1 as a co-receptor for $VEGF_{165}$ may enhance the angiogenic stimulus within the regenerating endometrium. Angiogenesis and endothelial growth occurring during the formation of the endometrium might be a result not only of VEGFR-2 and VEGF165 action, but also of NRP-1

co-expression. Since an appropriate remodelling of the human endometrium, including extensive angiogenesis processes, is mandatory for successful blastocyst implantation, the possibility of using a multiple range of receptors seems to be reasonable for angiogenic growth factors such as VEGF. A better understanding of the well-defined role of VEGF receptors in the normal reproductive cycle may also lead to new strategies in ameliorating reproductive abnormalities.

The section Acknowledgement was part of the corresponding Erratum.

Declaration of DM Baston-Büst´s role:

immunohistochemistry, interpretation of the data, writing of the manuscript

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Expression of the vascular endothelial growth factor receptor neuropilin-1 in the human endometrium

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Abstract

Angiogenesis is a key process in the endometrium which undergoes dramatic changes during the menstrual cycle. Molecules such as vascular endothelial growth factor (VEGF), acting via two tyrosine kinase family receptors (VEGFR1 [Flt-1] and VEGFR2 [KDR/Flk-1]), are potent modulators of angiogenesis and vascular remodelling in the endometrium. Recently, neuropilin-1 (NRP-1) was shown to be expressed in endothelial cells binding $VEGF_{165}$ and therewith enhancing the binding of $VEGF_{165}$ to VEGFR2. This suggests that NRP-1, in addition to the known VEGF receptors, may play an important role in VEGF-induced angiogenesis. In this study, the expression of NRP-1 in the cycling human endometrium has been investigated by reverse transcription (RT)-polymerase chain reaction (RT-PCR), semi-quantitative competitive RT-PCR (RT-cPCR) and immunohistochemical staining.

NRP-1 was expressed in all 32 endometrium samples throughout the menstrual cycle. However, samples from the proliferative phase showed significantly higher expression levels of NRP-1 mRNA compared to samples from the secretory phase (*t/c*-ratio 2.13 vs. 0.84, $p < 0.05$). Immunohistochemistry confirmed the results showing increased NRP-1 staining in vascular endothelium, glandular epithelium and stromal cells of the proliferative phase endometrium.

This study demonstrates mRNA and protein expression of NRP-1 in human endometrium samples throughout the menstrual cycle. The enhanced expression of NRP-1 in the proliferative phase suggests that it may participate in hormonally regulated changes of endometrial angiogenesis, preparing the endometrium for the implantation of an embryo. NRP-1 expression might act as a co-factor for $VEGF_{165}$ enhancing the angiogenic stimulus.

Keywords: Angiogenesis; Neuropilin-1; Endometrium; Menstrual cycle

1. Introduction

The endometrium and its vasculature undergo remarkable histologic and structural changes throughout the female menstrual cycle in preparation for embryonic implantation or subsequent shedding (Hess et al., 2007). These changes include tissue growth and remodelling as well as extensive angiogenesis of the pre-existing vessels (Lessey, 2002).

Based on Noyes et al. (1950), it has been appreciated that endometrial histology correlates directly with changes in the ovarian hormones estradiol (E_2) and progesterone (P). During the estrogen-dominated proliferative phase, the hormonal stimulus leads to an increase in cell proliferation. In contrast, during the

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progesterone-dominated secretory phase, a remodelling of the tissue, coiling of the spiral arterioles and growth of the subepithelial capillary plexus, occurs that prepares the endometrium for a possible implantation (Rogers and Gargett, 1998).

Angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for endometrial proliferation and regeneration during the menstrual cycle. It is a process normally only seen in fetal development, wound healing, metastasis and in the female reproductive tract (Folkman and Shing, 1992). The critical role of angiogenesis in endometrial function was shown in rodents when the angiogenesis inhibitor, AGM-1470, retarded growth of the endometrium when administered chronically to non-pregnant cycling mice (Klauber et al., 1997). While the ovarian steroids E_2 and P regulate the overall control of endometrial growth and regression, it appears unlikely that they are direct regulators of vascular growth.

Growth factors, such as vascular endothelial growth factor (VEGF), a potent modulator of angiogenesis and vascular remodelling, are expressed in the endometrium and seem to play a significant role in this process (Kruessel et al., 1999; Neufeld et al., 1999). During the menstrual cycle, expression of VEGF in the endometrium, particular in glandular epithelial and stromal cells, increases in the mid-secretory phase (Sugino et al., 2002). All five VEGF isoforms (Ferrara and Davis-Smyth, 1997) act via two tyrosine kinase family receptors: VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1) (Shibuya, 2006). Both receptors are localized mainly to vascular endothelial cells in human endometrium (Meduri et al., 2000).

Furthermore, cell surface receptor cross-linking studies show that VEGF also binds to other receptors than VEGFR-1 and -2 such as neuropilin-1 (NRP-1) (Soker et al., 1998) a membrane protein expressed in developing neurons regulating nerve fiber guidance (Kolodkin et al., 1997). Neuropilins are transmembrane glycoproteins of approximately 140 kDa with an extracellular portion containing three different structural domains, making it unlikely to function as independent receptors. NRP-1 is expressed in endothelial cells binding $VEGF_{165}$ and therewith enhancing the binding of $VEGF_{165}$ to VEGFR2 (Soker et al., 2002; Mac Gabhann and Popel, 2005). More recently, it was shown that NRP-1 not only enhances VEGF165 binding to VEGFR2 but also independently promotes cell signalling in endothelial cells (Wang et al., 2003).

Regarding the endometrium there is limited available information on NRP-1 expression. It was shown though to be involved in the regeneration process after menstrual shedding (Punyadeera et al., 2006). Furthermore, a study of human and rhesus macaque endometrium showed a high but stable expression of NRP-1 mRNA in vascular endothelium and stromal cells throughout the cycle and an elevated expression during the mid-to late proliferative phase in the luminal epithelium (Germeyer et al., 2005). These results suggest that, in addition to the classical tyrosine kinase receptors VEGFR1 and VEGFR2, VEGF also binds with high affinity to the alternative transmembrane receptor NRP-1. Therefore, NRP-1 may play an important role in VEGF-induced vascular permeability and angiogenesis required for the reproductive cycle and embryonic implantation. So far, little is known about expression of NRP-1 in human endometrium since most studies are conducted in the baboon and macaque model. Therefore, the aim of this study was to elucidate the expression pattern of NRP-1 in human proliferative versus secretory phase endometrium.

2. Material and methods

2.1. Uterus tissue specimens

Uterus samples were obtained from cycling women undergoing hysterectomy or endometrial biopsy for benign conditions, after written informed consent (approved by the University's ethical board). Samples were obtained from 32 subjects (22 early proliferative phase samples from day 4 to 8 of the cycle/10 mid-secretory phase samples from day19 to 24 of the cycle) between 26 and 42 years of age who had regular menstrual cycles (28–34 days) and normal hormonal profiles, were documented not to be pregnant, had no diagnosis of polycystic ovarian syndrome or endometriosis and no history of steroid hormone medications within three months of endometrial sampling. Half of each specimen was fixed in 10% paraformaldehyde (PFA) and embedded in paraffin for histological examination and the corresponding half directly processed for RNA extraction. The menstrual phase was determined by histological examination according to the criteria of Noyes et al. (1950) by two independent pathologists.

2.2. RNA extraction

Total RNA was extracted from tissue using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The RNA preparations were then DNase-treated and stored in diethylpyrocarbonat (DEPC)-treated water. Purity and quality were analyzed

by determining the absorbance ratio at 260/280 nm and 1.5% gel electrophoresis.

2.3. Reverse transcription reaction (RT)

For each endometrium sample (1 μg), 20 μl RT-mix was prepared (1 × PCR-buffer, 5 mM $MgCl_2$-solution, 2.5 μM oligo-dT_{16} (MWG Biotech AG, Ebersberg), dNTP-mix (1 mM each) with 1 U MuLV reverse transcriptase (all GeneAmp RNA PCR Core Kit, Applied Biosystems, Branchburg, NJ), DEPC-treated H_2O (20 μl) in a 0.5 ml PCR tube (Eppendorf, Hamburg)). The RT-reaction was carried out in the DNA thermal cycler using a program with the following parameters: 42 °C, 60 min; 99 °C, 5 min; 4 °C ∞. As a negative control, 1 μl of DEPC-H_2O was added to the RT-mix. After the reaction was completed samples were diluted 1:5 and stored in −20 °C.

2.4. Primers for polymerase chain reaction (PCR)

The cDNA sequences for β-actin and NRP-1 were obtained from the GenBank database of the National Centre for Biotechnology Information (NCBI) of the National Institute of Health (NIH) (http://www2.ncbi.nlm.nih.gov/). Primer sequences were selected using the program OLIGO 5.0 Primer Analysis Software (National Bioscience, Plymouth, USA). Primers were designed to cross intron/exon boundaries to ensure that the amplified product resulted from cDNA rather than contaminating genomic DNA. The identities of all PCR products were confirmed by cycle sequencing. The primer sequences, accession numbers and size of the amplified fragments are listed in Table 1.

2.5. Construction of the competitive cDNA fragments

Competitive cDNA fragments were constructed utilizing a method described previously (Raga et al., 1998). A 'floating' primer was designed for the cDNA to introduce an internal deletion, resulting in a competitive fragment with the same primer binding characteristics as the target cDNA (Fig. 1). Competitive cDNA was extracted from the agarose gel with an extraction kit (QIAEX II, Qiagen) and quantitated by spectophotometry. The sequences of the 'floating' primer and size of the resulting competitor fragment are listed in Table 1.

2.6. RT-polymerase chain reaction (RT-PCR)

Five microlitres of the RT-product were added to 45 μl of PCR-mastermix (1× PCR buffer, 1.75 mM $MgCl_2$ solution, 0.25 mM primer each (MWG Biotech AG), dNTP-mix (0.2 mM each), 1 U/rct of AmpliTaqGold®Polymerase (all Applied Biosystems), (50 μl) DEPC-treated H_2O. PCR was started in a DNA thermal cycler and heated to 99 °C for 15 min to activate the polymerase. After completion of 40 cycles of 94 °C for 45 s, annealing temperature (54 °C for β-actin and 56 °C for NRP-1) for 45 s and 72 °C for 60 s, the reaction was terminated at 72 °C for 5 min and cooled down to 4 °C. Agarose gel electrophoresis was carried out using β-actin as an internal standard.

2.7. Competitive polymerase chain reaction (RT-cPCR)

Five microlitres RT product were mixed with 40 μl PCR-mastermix containing 5 μl 10× reaction buffer; 0.2 μl Taq DNA polymerase (Amersham Pharmacia Biotech Inc., Piscataway, NJ); 2 μl dNTP- mix (50 mM each, Eppendorf); 2 μl of 5′ and 3′ specific primers (5 μM each), 31.8 μl H_2O and 0.1 amol/μl competitive cDNA for NRP-1 in 5 μl H_2O. The PCR was initiated by heating up to 94 °C for 3 min, followed by 32 cycles denaturation at 94 °C for 45 s, annealing at 56 °C for NRP-1 and extension at 72 °C for 45 s. The reaction was terminated at 72 °C for 5 min and cooled down to

Table 1
Primers used for RT-PCR.

mRNA	Accession number of mRNA	Size of amplified fragment (bp)	Type of primer	Sequence of primer
NRP-1 target	NM_003873	386	5′	5′-cccgcacctcattcctacatc-3′
			3′	5′-cattcatccaccaagttcccg-3′
NRP-1 competitor	NM_003873	262	5′ float	5′-cccgcacctcattcctacatcgctcggactggaagatgatc-3′
β-Actin	NM_12481	409	5′	5′-caa ggt gtg atg gtg gga atg g-3′
			3′	5′-cag gat ggc gtg agg gag ca-3′

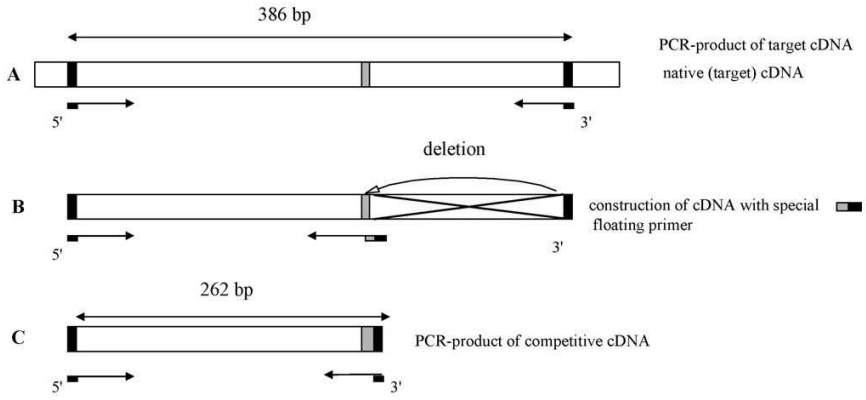

Fig. 1. (A) Size of target PCR product and location of the primer binding sites for the 3′ and 5′ primers (black), and the primer to construct the competitive cDNA (grey) on the native NRP cDNA. (B) Construction of the competitive cDNA. An artificial deletion is created to synthesize a shorter cDNA fragment with the same primer binding characteristics than the target cDNA. (C) Size of competitor-PCR product and location of the primer binding sites for the 3′ and 5′ primers (black).

4 °C. In each experiment, a negative control reaction in which no cDNA was added was included. Preliminary experiments were performed to determine the optimal numbers of PCR amplification cycles to ensure that the amplification was in the exponential phase and had not reached the plateau. PCR products were visualized on a 2% agarose gel and densitometrical analysis was carried using the GelDoc 1000 System (Bio-Rad Laboratories, Hercules, CA) with Molecular Analyst Software (Bio-Rad). Densitometrical values were used to calculate the ratios between target and competitor bands (*t*/*c*-ratio).

2.8. Immunohistochemistry

Tissues were fixed in 10% buffered formalin and subsequently embedded in paraffin. Sections were cut at 5 μm and mounted onto Superfrost Plus microscope slides (Fisher Scientific, Hanover Park, IL). Sections were deparaffinized in xylene (Fisher Scientific), rehydrated in graded ethanol concentrations and rinsed in phosphate-buffered saline. Tissues were subjected to antigen retrieval (Vector H3300, Burlingame) for 20 min. All slides were quenched in 0.3% (v/v) H_2O_2 and consecutively incubated with blocking serum for 20 min (Vector) followed by 1 h of incubation with a polyclonal primary antibody (AB) (15 μg/ml) (goat anti-rat NRP-1, R&D Systems, MN) at room temperature (rt). Although being a goat anti-rat AB against NRP-1 rather than an anti-human AB, the AB was applicable for staining of NRP-1 in human endometrium samples since the amino acid sequence of rat NRP-1 extra-

Table 2
Rating of the staining intensity of endometrial samples of both phases of the cycle.

	Gland	Vessel	Stroma	Perivascular stroma
Proliferative phase	+++	+++	++	+++
Secretory phase	−/+	++	+	+

The following symbols encode the staining intensity observed: (−/+), no to low staining; (+), low staining; (++), moderate staining and (+++), intense staining.

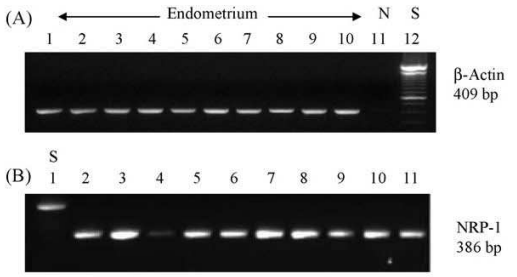

Fig. 2. Electrophoresis gels showing RT-PCR analysis for representative endometrium samples of both cycle phases for the housekeeping gene β-actin and the gene of interest NRP-1. (A) Slots 1–5 show samples from the proliferative phase and slots 6–10 from the secretory phase expressing β-actin as an internal standard showing the viability of the samples investigated showing bands at 409 base pairs (bp). The negative control (N) [H_2O] is represented in slot 11 and the standard bp ladder (S) in slot 12. (B) Shows the RT-PCR of representative endometrium samples (slots 2–11) from both cycle phases (slots 2–6 secretory phase samples, slots 7–11 proliferative phase samples) expressing NRP-1 at 386 bp. The position of the S represents slot 1.

3. Results

3.1. RT-PCR and competitive RT-PCR

From a total of 32 endometrial samples, all were positive for β-actin mRNA expression and therefore considered for further examination (Fig. 2A). We detected mRNA for NRP-1 in 32 out of 32 (100%) human endometrial samples, covering 22 samples from the proliferative phase of the cycle and 10 samples from the secretory phase of the cycle (Fig. 2B). Since all samples expressed NRP-1, a further step to quantitate the NRP-1 expression within the samples, and therewith gain more information about the expression pattern, was applied using the semiquantitative competitive PCR approach.

First, a dilution series was performed to find the best competitor concentration to work with. This dilution experiment revealed the working concentration of 0.1 amol/μl of competitor to be most suitable (Fig. 3A). In the following RT-cPCR experiments, the expression levels of NRP-1 were comparable as judged from the competitor used in the cPCR reactions (Fig. 3B). Samples from the proliferative phase of the cycle showed a significant higher expression level of NRP-1 mRNA compared to samples from the secretory phase (*t/c*-ratio 2.13 vs. 0.84) with a statistical significance of $p = 0.027$ (Fig. 3C).

3.2. Immunohistochemistry

Further, the expression of NRP-1 protein was evaluated using immunohistochemistry. Of the 32 samples investigated for mRNA expression, five samples of the proliferative and five samples of the secretory phase of the cycle were randomly chosen for further immunohistochemical analysis (AB R&D Systems AF 566). The procedure was repeated two times for several sections (minimum of two) from each chosen sample. The results were reproducible with very little variety in all samples investigated as confirmed by two independent investigators. Fig. 4A shows staining for NRP-1 protein in endometrium samples from human cycling endometrium in the proliferative phase and Fig. 4B in the secretory phase. The slides of the proliferative phase show strong NRP-1 immunoreactivity in the vascular endothelium (Vessel) [+++] as well as in the glandular epithelium (Gland) [+++]. Within the stromal cell compartment, the perivascular area showed the most staining [+++] followed by the remaining stromal compartment with a rather moderate staining [++] (Fig. 4A). In comparison to the proliferative phase, only a moderate staining [++] in the vascular endothelium followed by a low staining in the stromal compartment [+] and almost no staining [−/+] in the glandular epithelium of secretory phase endometrium occurred (Fig. 4B) (see Table 2).

4. Discussion

The regulation of human endometrial angiogenesis is a necessary prerequisite for a temporal and spatial limited change of the tissue construction in accordance to prepare the endometrium for an implanting conceptus or in its absence for a limited shedding. So far, many studies have focused on the endometrial expression of VEGF and its receptors VEGFR1 and VEGFR2 (Kruessel et al., 1999; Sharkey et al., 2000; Möller et

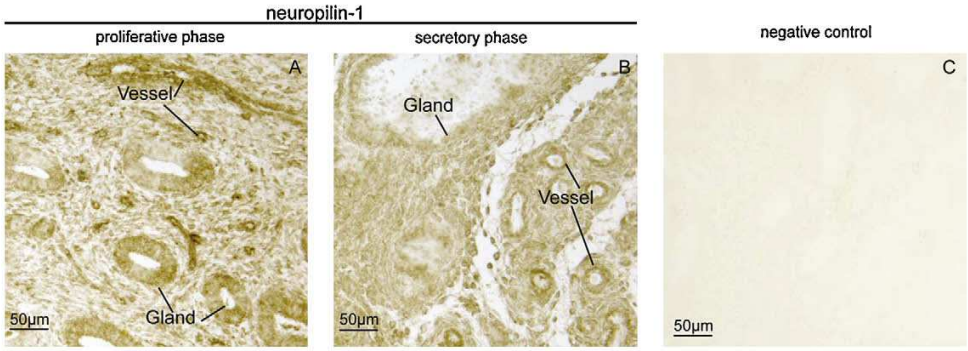

Fig. 4. Immunohistochemical staining for NRP-1 protein in endometrium samples from the early-proliferative phase (A), the mid-secretory phase (B) and negative control (C). In the proliferative phase NRP-1 immunoreactivity was strong in the vascular endothelium and glandular epithelium. The strongest activity in the stromal cell compartment occurred in the perivascular area whereas the remaining stroma showed a rather moderate staining. Whereas only a moderate staining in the vascular endothelium followed by a weaker staining in the stromal compartment and glandular epithelium of secretory phase endometrium occurred. Magnification ×200, the scale bar indicates 50 μm. In total five samples of each group were investigated with two repeats within each section chosen.

al., 2001) but a growing body of evidence suggests that endometrial angiogenesis is a complex process orchestrated by several different angiogenic and angiostatic factors (Cross and Claesson-Welsh, 2001; Hanahan and Folkman, 1996). This acknowledges pivotal roles for the known growth factors, such as VEGF and its receptors VEGFR1 and VEGFR2, but suggests as well that other factors such as neuropilins for example are necessary co-factors for efficient angiogenesis (Halder et al., 2000; Pavelock et al., 2001). Maas et al. (2001) investigated the angiogenic activity of endometrium applying the chick chorioallantoic membrane (CAM) assay. Their results showed angiogenic activity throughout the menstrual cycle, with a significant increased vascular density index in the early proliferative as well as early and late secretory phase compared to the late proliferative phase.

Since it is highly unlikely that one angiogenic factor alone is responsible for these complex changes in the endometrium, our study has addressed the potential regulation and expression of NRP-1, the major known neuropilin isoform facilitating VEGF action, throughout the human menstrual cycle. This study demonstrates mRNA and protein expression of NRP-1 in the human endometrium during the early proliferative and the mid-secretory phase of the natural menstrual cycle. The spatial distribution of NRP-1 expression seen in this study resembled the expression pattern of VEGFR-1 and VEGFR-2 found in other studies. For example, Möller et al. (2001) described VEGFR-1 and VEGFR-2 protein expression in and around endometrial blood vessels as well as in luminal and glandular epithelial cells throughout the cycle. This co-expression of NRP-1 and VEGFR-1 and -2 in the same endometrial cells supports the view of NRP-1 as a co-receptor for $VEGF_{165}$. Furthermore, the increased NRP-1-expression in the proliferative phase leads to the assumption that NRP-1 as a co-receptor for $VEGF_{165}$ may enhance the angiogenic stimulus within the regenerating endometrium. These mRNA data were further confirmed by the immunohistochemical finding that NRP-1 was also preferentially expressed in the proliferative phase samples. Furthermore, the most predominant staining in proliferative phase endometrium occurred in vascular endothelium, glandular epithelium and stromal cells surrounding the vasculature followed by a moderate staining of the remaining stroma. However, in secretory phase endometrium, only moderate staining in the vascular endothelium followed by a weaker staining of the stromal compartment and almost no staining of the glandular epithelium occurred. These findings suggest that VEGF via its receptor NRP-1 influences endothelial and epithelial cells as well as stromal cells, a point of view supported by a recent study proposing that VEGF regulates the function of endometrial stroma and glandular epithelial cells in an autocrine and paracrine fashion possibly to prolong the lifespan of those cells which might support a successful pregnancy (Sugino et al., 2002).

In contrast to these sets of data, Germeyer et al. (2005) found constant NRP-1 expression in stromal cells and vessels without a change during the cycle, whereas no staining at all of NRP-1 in the glandular epithelium was detected. Furthermore, they documented NRP-1 staining in the luminal epithelium of only proliferative phase endometrium samples. These differences might be explained by the fact that Germeyer et al. (2005) applied *in situ* hybridization techniques and therewith investigated the mRNA expression of NRP-1 in the cycling human endometrium compared to the present study where protein expression was investigated. However, an even predominant NRP-1 staining in the endometrial glands of the rat uterus was shown before by another group (Pavelock et al., 2001). Nevertheless, rat endometrium did not show staining of NRP-1 in stromal cells as found within this study, which suggest possible differences between species.

Thus, angiogenesis and endothelial growth occurring during the formation of the endometrium might be a result not only of VEGFR-2 and $VEGF_{165}$ action but also of NRP-1 co-expression. Since an appropriate remodelling of the human endometrium, including extensive angiogenesis processes, is mandatory for successful blastocyst implantation, the possibility of using a multiple range of receptors seems to be reasonable for angiogenic growth factors such as VEGF. A better understanding of the well-defined role of VEGF receptors in the normal reproductive cycle may also lead to new strategies in ameliorating reproductive abnormalities.

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Erratum

Erratum to "Expression of the vascular endothelial growth factor receptor neuropilin-1 in the human endometrium" [J. Reprod. Immunol. 79 (2009) 129–136]

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2.7 Expression of vascular endothelial growth factor receptor Neuropilin-1 at the human fetal-maternal interface (in resubmission to European Journal of Obstetrics, Gynecology and Reproductive Biology)

DM Baston-Büst, AC Porn, A Schanz, JS Krüssel, W Janni, AP Hess

Implantation of the human embryo into a receptive endometrium is the central process in the early fetal-maternal dialogue that leads to a successful pregnancy. Angiogenesis is a process which is required for both, the remodelling of endometrial tissue via embryonic invasion as well as for the supply of nutrition and oxygenation for the growing embryo. The complex molecular signalling cascades which lead to this process are not yet fully understood, but there is increasing evidence that cytokines and growth factors are involved in these early processes of embryonic implantation. VEGF is mitogenic for endothelial cells and stimulates cell differentiation, proliferation, migration and survival. Recent evidence shows that only the VEGF/VEGFR-2 signalling cascade plays the key role in the maintenance of early pregnancy through regulation of angiogenesis at the fetal-maternal interface. Moreover a discrepancy in the expression of angiogenic factors e.g. VEGF and the subsequent level of vascularisation between women with idiopathic recurrent spontaneous abortions and healthy controls was demonstrated. Another transmembrane protein, Neuropilin-1 (NRP-1) was characterized as an alternative receptor for VEGF. This receptor, a glycoprotein of approximately 140 kDa belongs to the semaphorin family, is expressed in developing neuronal cells and is responsible for neuronal cell guidance. Recently, the carboxyterminal peptide, DKPRR, was identified as the binding motif for NRP-1 in exon 8a of distinctive VEGF splice variants. The expression of NRP-1 within the human body is manifold which implicates its necessity in the regulation process of different signalling pathways.
Functioning as a receptor for VEGF underlines NRP-1s importance in the regulation of angiogenesis during embryonic implantation and pregnancy. The aim of this study was to elucidate the expression and localization of NRP-1 at the human fetal-maternal interface during the stages of early, mid and late gestation.

NRP-1 protein was clearly expressed in total cellular protein of all human choriocarcinoma cell-lines (JEG-3, Jar, BeWo). The analysis of total cellular protein of human cytotrophoblast (6, 17, 26 weeks of gestation) revealed a declining NRP-1 expression from first to third trimester samples.

The sections of the early stages (6. and 11. weeks of pregnancy) show a strong NRP-1 immunoreactivity in the decidual stroma [+++], glandular epithelium [+++], vascular endothelium [+++] as well as in the cytotrophoblast [+++]. In comparison to the early stages of pregnancy the samples of the 2. trimester only showed a moderate staining in the decidua [++], glandular epithelium [++] and vascular endothelium [++] whereas still an intensive staining of the cytotrophoblast [+++] occurred. In the third trimester samples, the expression of NRP-1 in the decidua declined to a moderate to low expression [++/+] whereas only a week staining in the cytotrophoblast remained [+/-].

The regulation of human angiogenesis at the fetal-maternal interface is an important precondition for the embryonic implantation or in its absence a possible reason for failure of this complex process. But there is a growing body of evidence, that the regulation of blood vessel growth rather relies upon a complex signalling cascade, affected by an array of angiogenic factors and receptors, as for example neuropilins. Because it is likely that an efficient angiogenesis is not just controlled by a single pair of growth factor and receptor, we investigated the expression of NRP-1 at the fetal-maternal interface during the embryonic implantation as well as in the course of pregnancy, as a co-receptor for the potent growth factor VEGF. In the first trimester, the formation and invasion of new blood vessels is one of the most important steps for an efficient implantation, whereas in the later pregnancy, the growing embryo is supplied via the placenta. This explains the high expression of NRP-1 in the early stages of pregnancy when extensive angiogenesis is essential for the embryos survival and its decline towards the third trimester, shown in this study. An earlier study supports this thesis as they presented a successful embryoid body angiogenesis assay based on murine stem cells with vessel-like structures by using $VEGF_{165}$ in an NRP-1 expressing model. The finding of a stronger NRP-1 expression in human proliferative

compared to secretory phase endometrium supports the results of this study suggesting an increase of NRP-1 synthesis in times of augmented demand for angiogenic processes. During implantation and placentation sufficient levels of VEGF and its receptors are required for angiogenesis and the embryo itself might activate its expressions as IL-1ß is a well-characterized early embryonic secretion product. Blocking of NRP-1 is additive to blocking of VEGF as a possible aim of new anti-angiogenic tumor therapies regarding tumor growth and vessel formation. The hypothesis that not only a single factor, e.g. VEGFR2, regulates angiogenesis but rather an interaction of various factors guarantee an efficient blood vessel growth, was supported by this study investigating the expression of NRP-1 at the fetal-maternal interface. This linked network supports a successful reproduction by providing an optimized situation of ligand, receptor and co-receptor resulting in a balanced angiogenesis. This knowledge may lead to an amelioration of strategies in the therapy of reproductive abnormalities in the future.

Declaration of DM Baston-Büst´s role:

immunohistochemistry, interpretation of the data, writing of the manuscript

Running title: Expression of Neuropilin-1 at the human fetal-maternal interface

Expression of the Vascular-Endothelial-Growth-Factor-Receptor Neuropilin-1 at the Human Fetal-Maternal Interface

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Condensation

The expression of the Vascular- Endothelial-Growth-Factor (VEGF) co-receptor Neuropilin-1 (NRP-1) in human decidua and trophoblast suggests an important role in angiogenesis at the fetal-maternal interface during embryonic implantation and placentation.

Abstract

Expression of the Vascular-Endothelial-Growth-Factor-Receptor Neuropilin-1 at the Human Fetal-Maternal Interface

Dunja M. Baston-Büst*, Anne C. Porn *, Andrea Schanz, Jan-S. Krüssel, Wolfgang Janni, Alexandra P. Hess

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Objective:

Angiogenesis is required for a successful implantation of the invading blastocyst. The vascular endothelial growth factor (VEGF) is known as an important key player of angiogenesis and vascular remodelling during the implantation process. VEGF acts via its well characterized receptors VEGFR1 and VEGFR2. Neuropilin-1 (NRP-1) was shown to play an additional role in the signalling process of angiogenesis in human endometrium. NRP-1 binds to $VEGF_{165}$ and therewith enhances the binding of $VEGF_{165}$ to VEGFR2. The expression of NRP-1 has been demonstrated in human endometrium during the menstrual cycle, leading to the hypothesis that NRP-1 might play a role in the regulation of endometrial angiogenesis and possibly furthermore during the vascular remodelling within embryo implantation.

Study design:

NRP-1 protein expression was investigated in human cell lines - choriocarcinoma (JEG-3 and Jar) and trophoblastic cell line (BeWo) aiming to evaluate the expression of NRP-1 *in vitro* - as well as in human decidua of all three trimesters of pregnancy by western blot analysis (n=3 samples each trimester of pregnancy). The localization of NRP-1 in human decidua of all three trimesters of pregnancy was analyzed by immunohistochemistry (n=5 samples each trimester of pregnancy).

Results:

NRP-1 protein was expressed in all cell lines examined. Corresponding to the analysis of human tissue by western blot, NRP-1 protein was higher expressed in the early pregnancy followed by a low to no expression at the end of pregnancy. NRP-1 was expressed in the decidua and villi of all samples investigated. Regarding the localization, the strongest expression of NRP-1 was shown in the vessels, stroma and glands of decidua as well as in the invading cytotrophoblast of first trimester samples, followed by a decline in expression towards the third trimester samples where only a moderate to low staining in the decidual compartment of NRP-1 remained, whereas no to low staining was found in the corresponding cytotrophoblast.

Conclusions:

This is the first study showing the expression of NRP-1 in human decidua and trophoblast suggesting an important role for the VEGF co-receptor NRP-1 besides the established receptor VEGFR2 at the fetal-maternal interface during the embryonic implantation and placentation.

Keywords: neuropilin-1 (NRP-1), vascular endothelial growth factor (VEGF), angiogenesis, fetal-maternal interface, implantation

1. Introduction

Implantation of the human embryo into a receptive endometrium is the central process in the early fetal-maternal dialogue that leads to a successful pregnancy (1). After leaving its protective zona pellucida, the embryonic trophoblast gets in direct contact with the endometrial epithelium and starts penetration of the epithelium to invade the endometrial stroma (2). Within the endometrial stroma, initiation of angiogenesis, the formation of new blood vessels from a pre-existing endothelium, is crucial (3). Angiogenesis is a process which is required for both, the remodelling of endometrial tissue via embryonic invasion as well as for the supply of nutrition and oxygenation for the growing embryo (4). The complex molecular signalling cascades which lead to this process are not yet fully understood, but there is increasing evidence that cytokines and growth factors are involved in these early processes of embryonic implantation (4-7). Vascular Endothelial Growth Factor (VEGF), a potent modulator of angiogenesis and vascular remodelling plays a critical role in the molecular mechanisms during menstrual cycle as well as in the process of invasion, implantation and embryonic growth (1, 4). VEGF is mitogenic for endothelial cells (8) and stimulates cell differentiation, proliferation, migration and survival (9). The VEGF gene-family is a ligand system, consisting of five isoforms resulting from an alternative splicing process of VEGF mRNA (10). All isoforms act via the transmembrane tyrosine kinase receptors VEGFR1, VEGFR2 and the soluble receptor (sflt) antagonizes VEGF (11). Recent evidence shows that only the VEGF/VEGFR-2 signalling cascade plays the key role in the maintenance of early pregnancy through regulation of angiogenesis at the fetal-maternal interface (12). In this study, an organ specific spectrum of impact and allocation demonstrating the importance of VEGFR2 in the VEGF regulated angiogenic process was shown since blocking of VEGFR-2 led to failure of embryonic implantation (12). Furthermore, there is evidence that a VEGF-polymorphism and haplotype can be a genetic determent for the risk of idiopathic recurrent spontaneous abortions (13). Moreover a discrepancy in the expression of angiogenic factors e.g. VEGF and the subsequent level of vascularisation between women with idiopathic recurrent spontaneous abortions and healthy controls was demonstrated (14). In addition to that, angiogenic growth factors seem to play an important role in the pathogenesis of diseases in pregnancy as preeclampsia, which is associated with shallow implantation and incomprehensive angiogenesis (15, 16). All these pathogenic mechanisms highlight the importance of a proper induction of angiogenesis in the early stage of embryonic implantation and underline the significance of understanding the expression of growth factors and their receptors at the fetal-maternal interface.

Another transmembrane protein, Neuropilin-1 (NRP-1) was characterized as an alternative receptor for VEGF (17). This receptor, a glycoprotein of approximately 140 kDa belongs to the semaphorin family, is expressed in developing neuronal cells and is responsible for neuronal cell guidance (17). NRP-1, consisting of an

extracellular unit and containing three different structural domains is also called a non-signalling co-receptor, which implicates its function as a receptor, operating in a dependent manner (18, 19). NRP-1 specifically binds $VEGF_{165}$ and therewith enhances the binding to VEGFR2 (17, 19, 20). Moreover NRP-1 likewise acts independently by promoting cell signalling in endothelial cells and by forming a complex with VEGFR1 and $VEGF_{121}$ (18, 21). Recently, the carboxyterminal peptide, DKPRR, was identified as the binding motif for NRP-1 in exon 8a of distinctive VEGF splice variants (22). Additionally a difference in kinetics of downstream targets of VEGF, e.g. p38 MAP kinase, depending on NRP-1 binding VEGF versus VEGF lacking NRP-1 binding was reported (23). The expression of NRP-1 within the human body is manifold which implicates its necessity in the regulation process of different signalling pathways. For instance NRP-1 is expressed by most T- regulatory cells (Treg) leading to a higher sensitivity to limiting amounts of antigen through prolonged interaction with immature dendritic cells (24). In the mouse model, NRP-1 displays a novel marker for Treg, which is upregulated at the fetal-maternal interface and therewith inducing a privileged tolerant microenvironment for the embryo (25, 26). The hypothesis that Tregs act as a protection against early abortion is supported by the findings that an upregulation of Tregs via heme oxygenase 1 prevented fetal rejection in the mouse (26). Functioning as a receptor for VEGF underlines NRP-1s importance in the regulation of angiogenesis during embryonic implantation and pregnancy. The aim of this study was to elucidate the expression and localization of NRP-1 at the human fetal-maternal interface during the stages of early, mid and late gestation.

2. Material and methods

Plan

Expression of NRP-1 in cycling human endometrium, led us to the hypothesis that this co-receptor could also be involved in angiogenesis at the human fetal-maternal interface during implantation. First, we focused on the detection of NRP-1 protein in human choriocarcinoma and trophoblast cell lines. Secondly, we examined the NRP-1 expression and localisation in samples of human trophoblast and decidua of all three trimesters of pregnancy.

2.1 Tissue Specimens

Decidua samples were obtained from pregnant women of all 3 trimesters, after written informed consent (approved by the University's ethical board). Samples were obtained from 8 subjects each from first to third trimester between 20-42 years of age, who had regular menstrual cycles (28-34 days) and normal hormonal profiles, had no diagnosis of polycystic ovarian syndrome or endometriosis and no history of steroid hormone medications within three months of endometrial sampling, undergoing termination of pregnancy. Three samples of each group were used for western blot analysis and 5 samples of each group for the immunohistochemistry approach. All specimens were fixed in 10 % paraformaldehyde (PFA) and embedded in paraffin for histological examination and immunohistochemistry.

2.2 Western blot

Total cellular protein was isolated from choriocarcinoma cell lines (JEG-3 and Jar) and trophoblast cell line (BeWo) (passage 5-7) as well as from cytotrophoblast cells of all three trimesters following acid guanidinium thiocyanate-phenol-chloroform extraction of RNA (27). Samples were resuspended in 1% SDS-solution. The concentration of protein was measured with Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, Illinois, USA).

30 µg total cellular protein was resuspended in 1 × sample loading buffer (Laemmli), heated to 95°C for 10 min and separated by discontinuous sodium dodecyl sulfate (SDS) -PAGE (12 %). The proteins were transferred to nitrocellulose membranes (Whatman ® Schleicher and Schuell, Dassel, Germany). Non-specific binding was blocked by Tris-buffered saline/0.1% Tween ® 20/5% fat-free dry milk solution for 1 h at rt (room temperature). For immunodetection, the membranes were incubated over night at 4°C with an anti-rabbit NRP-1 antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, California, USA) in Tris-buffered saline/0.1% Tween ® 20. Staining with ß-actin served as loading control. Then, the membranes were washed and incubated with

horseradish peroxidase (HRP) coupled anti-rabbit antibody (1:2000 dilution) (Amersham Biosciences, Freiburg, Germany) for 2 h at rt. The visualisation of proteins was accomplished by enhanced chemiluminescence (ECL Western blotting, Amersham Biosciences). Protein sizes were determined using Spectra™ Multi-color High Range Protein Ladder (Fermentas, St. Leon-Rot, Germany). On each blot, a control sample (JEG-3) was included. The blots were analyzed by the GelDoc 1000 system (Bio-Rad Laboratories, Hercules, CA, USA).

2.3 Immunohistochemistry

Sections were cut at 5 µm and mounted onto Superfrost Plus microscope slides (Fisher Scientific, Hanover Park, IL, USA). Sections were deparaffinized in xylene (Merck Chemicals, Darmstadt, Germany) and rehydrated in graded ethanol concentrations. Tissues were incubated with antigen retrieval (Vector Laboratories, Burlingame, CA, USA) for 20 min. Inhibition of internal peroxidase activity with 0.3 % H_2O_2 was followed by incubation with blocking serum for 20 min (Vector Laboratories). The sections were then incubated with the primary antibody (AB) goat anti-rat NRP-1 (R&D Systems, Minneapolis, MN, USA), mouse anti-human cytokeratin (Abcam, Cambridge, MA, USA), mouse anti-human von Willebrand factor (vWF) (Dako North America Inc., Carpinteria, CA, USA), rabbit anti-human Vascular endothelia growth factor (VEGF) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at rt, washed and incubated for 30 min with the matching biotinylated secondary AB (Vector Laboratories) at rt according to the manufacturer´s manual. Slides were incubated for another 30 min with Vectastain® Elite ABC reagent (Vector Laboratories). Visualization of staining was performed by immersing sections in peroxidase substrate solution (DAB; Vector Laboratories). Slides were analyzed and photographed with a Leica CTR 5000 upright microscope (Leica, Solms, Germany). Negative control experiments included incubation with non-specific goat IgG or missing primary or secondary antibodies.

2.3 Statistical analysis

The intensity of the immunohistochemical staining of the pregnancy samples was rated by two independent investigators (see Table I).

3. Results

NRP-1 protein was clearly expressed in total cellular protein of both human choriocarcinoma cell-lines (JEG-3, Jar) and of human trophoblast cell line (BeWo).

The analysis of total cellular protein of human cytotrophoblast (6, 17, 26 weeks of gestation) revealed a declining NRP-1 expression from first to third trimester samples.

The expression of NRP-1 protein in the fetal-maternal interface during all three trimesters of pregnancy was evaluated using immunohistochemistry. The procedure was repeated twice for several sections (minimum of three) from each chosen sample. The results specified in the following paragraph were reproducible with very little variety in all samples investigated as confirmed by two independent investigators. Figure 1 shows staining for NRP-1 protein in human fetal-maternal interface samples from the early (6. week of gestation, A-E) and late first trimester (11. week of gestation, F-J), second trimester (18. week of gestation, K-O) and the third trimester of pregnancy (29. weeks of gestation, P-T). The sections of the early stages (6. and 11. weeks of pregnancy) show a strong NRP-1 immunoreactivity in the decidual stroma [+++], glandular epithelium [+++], vascular endothelium [+++] as well as in the cytotrophoblast [+++]. In comparison to the early stages of pregnancy the samples of the 2. trimester only showed a moderate staining in the decidua [++], glandular epithelium [++] and vascular endothelium [++] whereas still an intensive staining of the cytotrophoblast [+++] occurred. In the third trimester samples, the expression of NRP-1 in the decidua declined to a moderate to low expression [++/+] whereas only a week staining in the cytotrophoblast remained [+/-]. The sections B and G show the immunoreactivity of VEGF, which is strong in the first trimesters decidual vessels and stromal core [+++] as well as in the cytotrophoblast [+++] and moderate in the stromal glands [++]. Whereas in the second (L) and third trimester (Q) a strong staining remained in the stroma and vessels [+++], followed by a moderate staining in the glands and a low to no staining of the cytotrophoblast [+/-].

4. Comments

The regulation of human angiogenesis at the fetal-maternal interface is an important precondition for the embryonic implantation or in its absence a possible reason for failure of this complex process. As of today, well characterized angiogenic growth factors, such as VEGF and its receptors VEGFR1 and VEGFR2 are known key players in human angiogenesis (28-30). But there is a growing body of evidence, that the regulation of blood vessel growth rather relies upon a complex signalling cascade, affected by an array of angiogenic factors and receptors, as for example neuropilins (30). This complex network of interconnecting growth factors and receptors demonstrates the importance of angiogenesis for the monthly assembling and changes in endometrial tissue as well as nutrition and oxygen supply for the developing embryo at the fetal-maternal interface. Because it is likely that an efficient angiogenesis is not just controlled by a single pair of growth factor and receptor, we investigated the expression of NRP-1 at the fetal-maternal interface during the embryonic implantation as well as in the course of pregnancy, as a co-receptor for the potent growth factor VEGF.

Herein, we could demonstrate a strong expression of NRP-1 in the choriocarcinoma cell lines and a trophoblastic cell line *in vitro*. The results of the analysis of total protein isolated from trophoblastic tissue from all three trimesters were consistent with the immunohistochemistry of the tissue of the fetal-maternal interface, with the highest expression in the early stages of pregnancy. (31)

In the first trimester, the formation and invasion of new blood vessels is one of the most important steps for an efficient implantation, whereas in the later pregnancy, the growing embryo is supplied via the placenta (4). This explains the high expression of NRP-1 in the early stages of pregnancy when extensive angiogenesis is essential for the embryos survival and its decline towards the third trimester, shown in this study. An earlier study supports this thesis as they presented a successful embryoid body angiogenesis assay based on murine stem cells with vessel-like structures by using $VEGF_{165}$ in an NRP-1 expressing model (23). The finding of a stronger NRP-1 expression in human proliferative compared to secretory phase endometrium supports the results of this study suggesting an increase of NRP-1 synthesis in times of augmented demand for angiogenic processes (19). Recently, NRP-1 was found to be increased on mRNA and protein level in isolated decidual stromal cells incubated with IL-1ß (32) These findings could be confirmed in decidua from women with histological intra-amniotic infection giving a hint that increasing VEGF and NRP-1 expression might lead to increased leukocyte migration being linked with preterm birth in late gestation (32). On the opposite, during implantation and placentation sufficient levels of VEGF and its receptors are required for angiogenesis and the embryo itself might activate its expressions as IL-1ß is a well-characterized early embryonic secretion product (33).

Furthermore, blocking of NRP-1 is additive to blocking of VEGF as a possible aim of new anti-angiogenic tumor therapies regarding tumor growth and vessel formation (34).

There is evidence that NRP-1 also plays an important role within various other aspects of the reproductive cycle. NRP-1 was identified on Treg which suggests an additional role besides the regulation of angiogenesis in the regulation of the immune system (24). In this context, an upregulation of NRP-1 as a marker of T-cells preparing a privileged surrounding for the implanting embryo in the mouse model was addressed (26). Another study in human showed that Treg and therewith NRP-1 are present at the fetal-maternal interface already in the first days of pregnancy (35). For a successful implantation and maintenance of pregnancy, acceptance of the semiallogenic embryo by the maternal immune system is mandatory. Within this system, Treg seem to play an important role in orchestrating immune tolerance towards the embryo (35). In this study, they showed a decline of mRNA of NRP-1, as well as interleukins in patients with miscarriage. There is further evidence that in absence of these growth factors, receptors and cytokines the implantation process is endangered possibly leading to spontaneous miscarriage (35). Overall, these studies demonstrated besides NRP-1s' established function in the reproductive cycle a further role during the implantation process and pregnancy which seems even more diverse and not limited to angiogenesis but also in modulating the immune tolerance towards the embryo.

The hypothesis that not only a single factor, e.g. VEGFR2, regulates angiogenesis but rather an interaction of various factors guarantee an efficient blood vessel growth, was supported by this study investigating the expression of NRP-1 at the fetal-maternal interface. This linked network supports a successful reproduction by providing an optimized situation of ligand, receptor and co-receptor resulting in a balanced angiogenesis. This knowledge may lead to an amelioration of strategies in the therapy of reproductive abnormalities in the future.

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Legends

	Decidua/Gland		Decidua/Vessel		Decidua/Stroma		Decidua / Cytotrophoblast	
	NRP-1	VEGF	NRP-1	VEGF	NRP-1	VEGF	NRP-1	VEGF
First trimester	+++	++	+++	+++	+++	+++	+++	++
Second trimester	++	++	++	+++	++	+++	+++	+/-
Third trimester	++/+	++	++/+	+++	++/+	+++	-/+	+/-

Table I

Rating of the staining intensity of decidua and villi samples of all trimesters of pregnancy. The following symbols encode the staining intensity observed. +/-: no to low, +: low, ++: moderate and +++: intense staining.

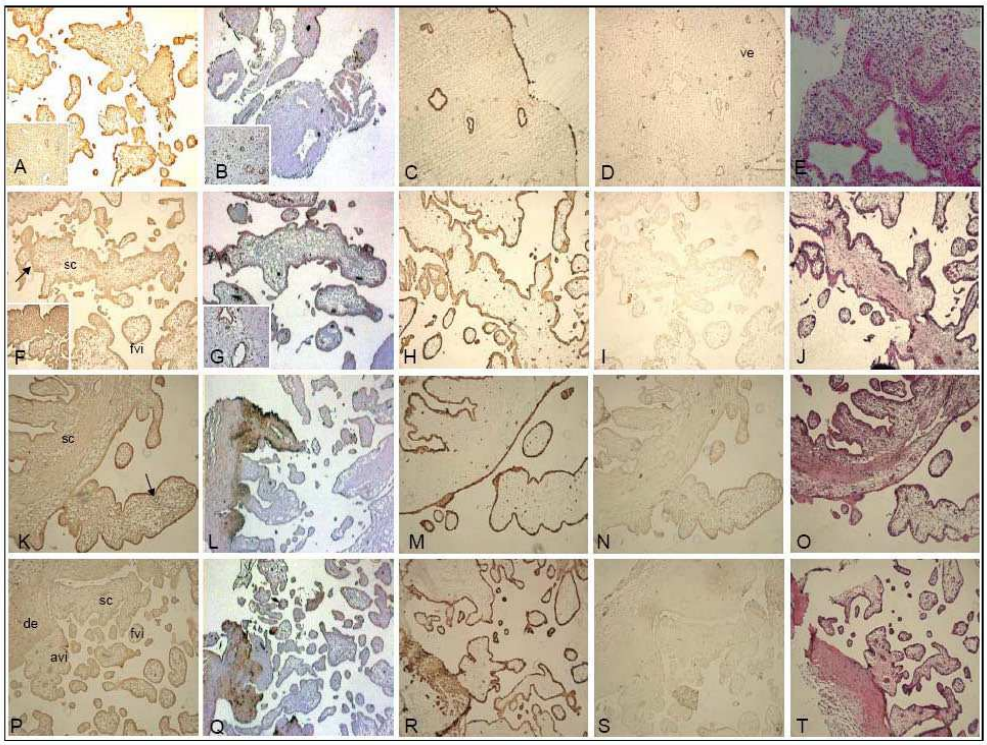

Figure 1

Immunohistochemical analysis of NRP-1 protein expression in human decidua and trophoblast during all three trimesters of pregnancy. The pictures A-E represent samples of the 6th week of gestation (early first trimester). A, B, F and G show corresponding villi to the deciduas in the inserts. Samples F-J originate of the 11th week of pregnancy (late first trimester), samples K-O from the 17th week (second trimester), and P-T demonstrate samples of the 26th week of pregnancy (third trimester). Magnification 630×. Samples A, F, K and P are stained with anti-NRP-1 antibody; samples B, G, L, Q with anti-VEGF; samples C, H, M, R with anti-cytokeratin as an epithelial marker; D, I, N, S with anti-vWF as a vessel marker and samples E, J, O and T are stained with haematoxylin-eosin (HE).

de = decidua, gl = gland, ve = vessel, vi = villus, arrow = cytotrophoblast, sc = stromal core, fvi = floating villus, avi = anchoring villus.

Figure 2

Western blot protein analysis of NRP-1 (130 kDa) expression of isolated total cellular protein samples of A) human chorion carcinoma (JEG-3, Jar) and trophoblast (BeWo) cell lines as well as of B) isolated human cytotrophoblast samples of all three trimesters (6, 17, 26 weeks of gestation). JAR protein served as a positive control.

A total number of 9 samples was investigated in each group. Figure 2 B shows one representative sample of each group.

3. Summary

The successful implantation of an embryo depends on a synchronized molecular embryo-maternal dialogue enabling the necessary modifications of maternal tissue as well as maturation and invasion of the embryo. The studies summarized in this work present cytokines, chemokines, angiogenic factors, proteinases, receptors, co-recectors and inhibitors involved in the complex network of interactions at the embryo-maternal interface. On the one hand, studies focussed on the embryo human and murine: describing the expression of the VEGF system as potent modulators of angiogenesis, the localization of the NRP-1 expression in the cytotrophoblast and the expression of proteinase inhibitors, cystatins, in order to regulate embryonic invasion. On the other hand, investigations focussed on factors expressed and regulating the maternal reproductive tract including fallopian tube and decidua were presented including the expression of the pro-invasive CTSS during secretory phase, the temporal but no spatial different expression of the members of the IL-1 system in the fallopian tube, the increase of NRP-1 expression during proliferative phase endometrium preparing angiogenesis for an implantation, the MAPK signalling cascade for the mediation of CXCL1 expression after contact with the embryo surrogate IL-1ß and the changes of chemokine and angiogenic factors profiling after knock-down of the chemokine co-receptor Sdc-1 in decidua *in vitro*.

All of these findings underline the complexity of molecules involved in the embryo-maternal dialogue enabling a proper implantation and subsequent development of a successful pregnancy.

4. Scientific *Curriculum Vitae*

Profession

08/2005 - today PhD student at the department of OB/GYN and Reproductive medicine at the Heinrich-Heine University medical center

Membership

German Society for Reproductive Medicine (Deutsche Gesellschaft für Reproduktionsmedizin, DGRM) (since 2006)
European Society of Human Reproduction and Embryology (ESHRE) (since 2010)

Articles in reviewed journals

- **"The effect of relaxin on the oxytocin receptor in human uterine smooth muscle cells."** U Friebe-Hoffmann, DM Baston, JP Chiao, LD Winebrenner, JS Krüssel, TK Hoffmann, J Hirchenhain, PN Rauk. Regulatory Peptides (2007), 138, 74-81.
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- **"CXCL1 expression in human decidua *in vitro* is mainly mediated via MAPK signalling cascade."** DM Baston-Büst, D Schuldt, W Janni, JS Krüssel, AP Hess. submitted to Journal of Clinical Endocrinology and Metabolism
- **"Syndecan-1 knock-down in decidualized human endometrial stromal cells leads to significant changes in cytokine and angiogenic factor expression patterns."** DM Baston-Büst, M Götte, A Schanz, W Janni, JS Krüssel, AP Hess. submitted to Reproductive Biology and Endocrinology
- **"Expression of the Vascular-Endothelial-Growth-Factor-Receptor Neuropilin-1 at the Human Fetal-Maternal Interface."** Dunja M. Baston-Büst*, Anne C. Porn *, Andrea Schanz, Jan-S. Krüssel, Wolfgang Janni, Alexandra P. Hess (*shared 1st authorship). In Resubmission European Journal of Obstretrics, Gynecology and Reproductive Biology

Oral presentations (o) & Posters (p)

- U Friebe-Hoffmann, J Hirchenhain, DM Baston. **Effekte von Relaxin auf die Signalkaskade des humanen Oxytozinrezeptors sowie das Cyclooxygenasesystem in Hinblick auf Weheninduktion.** Geburtsh Frauenheilk 2006; S149, PO 03.28 / 56. Annual Meeting of the German Society of Gynecology and Obstetrics (DGGG), Berlin, 2006 (p)

- AP Hess, J Hirchenhain, A Schanz, DM Baston, M Stoff-Khalili, B Mikat-Drozdzynski, U Friebe-Hoffmann, JS Krüssel. **Neuropilin-1, ein alternativer Rezeptor für Vascular Endothelial Growth Factor im menschlichen Endometrium.** J Reproductionsmed Endokrinol 2006; 3 (5), 344-345. / 26. Annual Meeting of the German Society of Reproductive Medicine (DGRM) (p)

- DM Baston, J Hirchenhain, AP Hess, JS Krüssel, U Friebe-Hoffmann. **Effekte von Relaxin auf die Signalkaskade des humanen Oxytozinrezeptors sowie des Cyclo-oxygenasesystems in Hinblick auf Weheninduktion.** J Reproductionsmed Endokrinol 2006; 3 (5), 345-346. / 26. Annual Meeting of the German Society of Reproductive Medicine (DGRM) (p)

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6. Statement

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den

Dunja Maria Baston-Büst