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.

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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

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; H₂O), 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 (E₂)- and progesterone (P₄)-dominated phase in female mice (magnification $\times 400$). 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 $\times 100$ (small inserts) and $\times 450$. 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 immuno-competent 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 $\times 100$ and $\times 400$ as indicated. The squares indicate the parts shown in $400\times$ 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 fetomaternal 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 a 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 CTSS 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 fetomaternal 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% CO₂. 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 μ l RT-mastermix was prepared (2.5 mM MgCl₂, 1 $\times$ RT buffer, dNTPs each 1 mM, 1.25 mM oligo d(T)₁₆, diethylpyrocarbonate (DEPC)-treated dH₂O ad 18.2 μ l; 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 μ l 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 dH₂O ad 50 μ l and stored at -20 °C until the PCR reaction was carried out.

DEPC-treated H₂O 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 μ l of diluted RT product was added to 45 μ l of specific PCR-1-mix for β -actin, *Ctss*, *Cst3*, *Cst7*, and *Il6* (2 mM MgCl₂, 1 $\times$ PCR buffer, 1 $\times$ Q-solution, dNTPs each 0.2 mM, outer primer pair each 0.3 μ M, 2.5 U/rct HotStarTaq Plus DNA polymerase, DEPC-treated dH₂O ad 45 μ l; 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')
<i>Ctss</i> out	AF038546.1	660	5'	5'-cct acc aag tgg gca tga ac-3'
			3'	5'-gcc atc aag agt ccc ata gc-3'
<i>Ctss</i> in		240	5'	5'-taa tcg gac att gcc tga ca-3'
			3'	5'-ctg gaa agc ttc ggt cat gt-3'
<i>Cst3</i> out	NM_009976	482	5'	5'-tcg ctg tga gcg agt aca ac-3'
			3'	5'-cat ggc agg tac tgc aag aa-3'
<i>Cst3</i> in		206	5'	5'-tgg tga gag ctc gta agc ag-3'
			3'	5'-tgc agc tga att ttg tca gg-3'
<i>Cst7</i> out	NM_009977.2	479	5'	5'-ggg cct gga gct gta ctt gc-3'
			3'	5'-aga gga gaa cag gca cct ca-3'
<i>Cst7</i> in		89	5'	5'-atg cat cac caa ctg gac aa-3'
			3'	5'-ggg atg acc cag act tca ga-3'
<i>Il6</i> out	NM_031168	339	5'	5'-gtt ctc tgg gaa atc gtg ga-3'
			3'	5'-gga aat tgg ggt agg ga-3'
<i>Il6</i> 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 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 μ l of the first-round PCR products were added to 45 μ l PCR-2-mix (2 mM MgCl₂, 1 $\times$ PCR buffer, 1 $\times$ Q-solution, dNTPs each 0.2 mM, inner primer-pair each 0.3 μ M, 2.5 U/ μ l HotStarTaq Plus DNA polymerase, DEPC-treated dH₂O ad 45 μ l; 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 μ g/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% H₂O₂ 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 LymphoPrep™ (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 μ m meshes, cell numbers counted, centrifuged, and resuspended in PBS with 0.5% FBS. The staining for cytometrical analysis started with 10⁵ 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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