SOCS genes expression during physiological and perturbed implantation in bovine endometrium

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Abstract

In mammals, suppressor of cytokine signalling (CISH, SOCS1 to SOCS7) factors control signalling pathways involved in the regulation of numerous physiological processes including pregnancy. In order to gain new insights into the biological functions of SOCS in the endometrium, a comprehensive analysis of *SOCS* gene expression was carried out in bovine caruncular (CAR) and intercaruncular (ICAR) tissues collected i) during the oestrous cycle, ii) at the time of maternal recognition of pregnancy and at implantation in inseminated females, iii) following uterine interferon-tau (IFNT) infusion at day 14 post-oestrus, iv) following a period of controlled intravaginal progesterone release and v) following transfer of embryos by somatic-cell nuclear transfer (SCNT). The regulatory effects of IFNT on *in vitro* cultured epithelial and stromal cells were also examined. Altogether, our data showed that *CISH*, *SOCS4*, *SOCS5* and *SOCS7* mRNA levels were poorly affected during luteolysis and pregnancy. In contrast, *SOCS1*, *SOCS2*, *SOCS3* and *SOCS6* mRNA levels were strongly up-regulated at implantation (day 20 of pregnancy). Experimental *in vitro* and *in vivo* models demonstrated that only *CISH*, *SOCS1*, *SOCS2* and *SOCS3* were IFNT-induced genes. Immunohistochemistry showed an intense SOCS3 and SOCS6 staining in the nucleus of luminal and glandular epithelium and of stromal cells of pregnant endometrium. Finally, *SOCS3* expression was significantly increased in SCNT pregnancies in keeping with the altered immune function previously reported in this model of compromised implantation. Collectively, our data suggest that spatio-temporal changes in endometrial *SOCS* gene expression reflect the acquisition of receptivity, maternal recognition of pregnancy and implantation.

Free French abstract

A French translation of this abstract is freely available at <http://www.reproduction-online.org/content/148/6/545/suppl/DC1>.

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Introduction

The *in utero* development and subsequent birth of a healthy offspring involve a succession of complex biological steps that have to be passed successfully. One major checkpoint is the process of implantation that relies on the coordinated interactions between a developmentally competent conceptus and the endometrium, the receptivity of which is controlled by progesterone (P₄) in mammals (Wang & Dey 2006, Spencer *et al.* 2007). At implantation, endometrial gene expression patterns reflect a combination of several biological processes including the actions of P₄, the impact of embryo-secreted factors and cellular interactions between the trophoblast and the luminal endometrial epithelium. Thus, the biological functions of the endometrium can be subdivided as follows: i) the

ability to respond differentially to embryos displaying various potentials to develop to term and ii) the ability to affect the development trajectory of the embryo at the epigenetic level, making this tissue an active and dynamic interface combining biosensor and biodriver properties (Sandra *et al.* 2011).

In cattle, implantation takes place from 19 to 20 days after oestrus (Guillomot 1995). In this species as in other ruminants, the extra-embryonic tissues first elongate before they attach to the endometrium. In ruminants, endometrial morphology differs from other mammalian species with the presence of caruncular (CAR) and intercaruncular areas (ICAR), both essential for supporting pregnancy. CAR areas are aglandular structures present in the cyclic endometrium, and they fuse with the foetal cotyledons to give rise to placentomes

(Atkinson *et al.* 1984). ICAR areas contain endometrial glands whose presence is critical for conceptus elongation (Gray *et al.* 2001, 2002). Recent high-throughput data have highlighted specific molecular pathways and genes that account for the biological functions related with each endometrial area (Mansouri-Attia *et al.* 2009a, Walker *et al.* 2010).

The elongation process of the extra-embryonic tissues is associated with the secretion of IFNT, which is exclusively produced by trophoblast cells during the peri-attachment period (Roberts 2007, Ealy & Yang 2009, Bazer *et al.* 2010). IFNT is considered the major pregnancy recognition signal in ruminants and its biological actions on the endometrium have been widely investigated (Roberts 2007, Spencer *et al.* 2007, Spencer *et al.* 2008). The major roles for IFNT include the inhibition of uterine prostaglandin (PG) F₂ α secretion, therefore preventing luteolysis, and the modification of the innate immune response (Hansen 2011). In sheep and cattle, IFNT intrauterine infusions, as well as *in vitro* cultures of endometrial cells incubated with IFNT, have allowed the identification of target genes that cover a wide range of biological processes and underlie the essential functions of this factor in the establishment of pregnancy (Choi *et al.* 2001, Kim *et al.* 2003, Gray *et al.* 2006, Forde *et al.* 2011a, 2012, Bauersachs *et al.* 2012, Eozenou *et al.* 2012, Mansouri-Attia *et al.* 2012, Oliveira *et al.* 2012).

Among the signalling cascades contributing to the regulation of endometrial physiology, a number of analyses have highlighted the JAK/STAT transduction pathway (Choi *et al.* 2001, Catalano *et al.* 2005, Joyce *et al.* 2007, Maj & Chelmonska-Soyta 2007, Spencer *et al.* 2007). As regulators of the JAK/STAT signalling cascade, the suppressor of cytokine signalling (SOCS) proteins control the amplitude and the duration of this signalling pathway (Rico-Bautista *et al.* 2006, Yoshimura *et al.* 2007). The SOCS family includes eight proteins namely the cytokine-inducible Src homology 2 (SH2)-domain containing protein (CISH) and SOCS1–7 that share a central SH2 domain, flanked by a variable *n*-terminal domain and a *c*-terminal SOCS box (Yoshimura *et al.* 1995, Endo *et al.* 1997, Naka *et al.* 1997, Starr *et al.* 1997, Hilton *et al.* 1998, Bullock *et al.* 2007). The SOCS proteins have been shown to inhibit the JAK tyrosine kinase activity, by competing with STAT transcription factors for phosphorylated tyrosine residues on cytokine receptors or by targeting bound signalling proteins for proteasomal degradation (Kile *et al.* 2002). The most studied members have been CISH, SOCS1 and SOCS3, which are rapidly but transiently induced by numerous local factors and hormones through the JAK/STAT pathway (Fujimoto & Naka 2003). The members of the SOCS family have been identified in numerous vertebrate and some invertebrate species (Delgado-Ortega *et al.* 2013) and are involved in the control of many biological processes and physiological functions including pregnancy (Yoshimura *et al.* 2007, Fitzgerald

et al. 2009). In the context of the uterus, SOCS1 and SOCS3 have been shown to contribute to decidualisation and uterine receptivity in the human (Dimitriadis *et al.* 2006, Aghajanova *et al.* 2009) and in the sheep, the endometrial expression of CISH, SOCS1, SOCS2 and SOCS3 transcripts has been reported to be affected by the presence of the conceptus (Sandra *et al.* 2005). Nevertheless, these data are scattered and fail to provide a comprehensive view of how SOCS factors take part in the regulation of endometrial physiology in mammals. The current study was designed to gain new insights into the involvement of the eight members of the SOCS family in the endometrium. Specifically, the main aims were to investigate i) the expression of steady state levels of the SOCS transcripts in cyclic and pregnant endometrial tissues as well as the cellular localisation of SOCS3 and SOCS6 protein, ii) the regulation of SOCS mRNA expression by P₄, a steroid hormone critical for the maintenance of pregnancy in mammals, and IFNT, the major signal of pregnancy recognition in ruminants, using *in vivo* and *in vitro* experimental models, and iii) the regulation of the SOCS transcripts in an experimental model of compromised implantation based on the inappropriate biological response of the endometrium facing bovine concepti produced by somatic-cell nuclear transfer (SCNT; Mansouri-Attia *et al.* 2009b).

Materials and methods

Animals

Animal care and all experimental procedures were completed in accordance with European Community Directive 86/609/EC, the Animal Research Ethics Committee of University College Dublin and the French Ministry of Agriculture (authorisation B91332). The protocols were registered by the Department of Health and Children (Ireland) or by the Regional Ethical Committee of Animal Experimentation of INRA and AgroParisTech (France, protocol 12-124). *In vitro* embryo production and embryo transfer protocols were registered by the French Veterinary Services (N°FRPB780 and FRTB910).

Experiment 1: regulation of SOCS mRNA expression during maternal recognition of pregnancy

As previously described (Forde *et al.* 2011b, Eozenou *et al.* 2012), synchronised cross-bred beef heifers were artificially inseminated to generate the pregnant group or were left as noninseminated to generate a cyclic group. The cyclic females were slaughtered at day 16 (*n*=5) and at day 20 (*n*=6) and uteri were immediately retrieved and flushed. A similar procedure was applied to inseminated heifers that were considered as pregnant (day 16, *n*=4; day 20, *n*=5) when the stage of conceptus development was consistent with the day of pregnancy as determined by stereomicroscopy (Degrelle *et al.* 2005). Based on ovarian morphology (Arosh *et al.* 2002), all cyclic heifers sampled at day 20 after oestrus presented a

regressed corpus luteum, whereas cyclic and pregnant heifers sampled at day 16 as well as pregnant heifers sampled at day 20 presented a functional corpus luteum. Endometrial CAR and ICAR areas were dissected separately from the uterine horn ipsilateral to the corpus luteum (Mansouri-Attia *et al.* 2009a), frozen in liquid nitrogen and then stored at -80°C for further analyses.

Experiment 2: endometrial SOCS mRNA expression at implantation

Charolais cows were synchronised by the Crestar method (Mansouri-Attia *et al.* 2009b). As described in Experiment 1, a cyclic group and a pregnant group (confirmed by the presence of a conceptus at the appropriate stage of development) were generated (cyclic: $n=5$, pregnant: $n=6$). Upon slaughter at day 20 after oestrus, tissue samples were immediately frozen in liquid nitrogen and stored at -80°C or fixed in 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) in PBS (Euromedex, Souffelweyersheim, France) for further analyses.

Experiment 3: impact of short-term IFNT uterine infusion on endometrial SOCS mRNA expression

Cyclic Charolais cows were synchronised by the Crestar method (Mansouri-Attia *et al.* 2009a). At day 14 after oestrus, recombinant ovine IFNT (roIFNT; 200 $\mu\text{g}/\text{ml}$, 25 ml/horn ; Sandra *et al.* 2005) or control solution (saline buffer) was infused into the uterine lumen, as previously reported (Forde *et al.* 2011a, 2012, Eozenou *et al.* 2012). The cows were slaughtered 2 h after the intra-uterine infusion and the endometrium of five IFNT-infused and five control cows was collected, dissected and stored as described in Experiment 2.

Experiment 4: impact of in vivo P_4 supplementation on the endometrial expression of SOCS mRNA in cyclic heifers

As previously described (Carter *et al.* 2008, Forde *et al.* 2011a, Eozenou *et al.* 2012), cyclic cross-bred heifers were implanted a P_4 -releasing intravaginal device containing 1.55 g of P_4 (Ceva Animal Health Ltd., Amersham, UK) on day 3 after oestrus. The females were slaughtered after 2 days (day 5 post-oestrus, normal P_4 , $n=4$; high P_4 , $n=3$) and 13 days (day 16 post-oestrus, normal P_4 , $n=3$; high P_4 , $n=3$) of P_4 supplementation. The strips of endometrium (containing CAR and ICAR areas) were collected, snap-frozen in liquid nitrogen and then stored at -80°C for further analyses.

Experiment 5: impact of bovine SCNT concepti on the endometrial SOCS expression

The cyclic Holstein heifers were synchronised using the Crestar method. As previously described (Mansouri-Attia *et al.* 2009b), heifers were separated in two experimental groups generated by artificial insemination (pregnant control group) or by transfer of SCNT embryos (two embryos into the ipsilateral horn to the corpus luteum). SCNT embryos produced with an adult donor cell line derived from a Holstein heifer (reference 5538; Degrelle *et al.* 2012). Upon slaughtering at day 20 post-oestrus, uteri were collected, concepti were

recovered by uterine flushing and the correct stage of development was confirmed by microscopy (Degrelle *et al.* 2005). Only females with a pregnancy confirmed by the presence of an appropriately developed conceptus were analysed (artificially inseminated, $n=4$; SCNT, $n=4$). CAR and ICAR endometrial areas were dissected and stored as described for Experiment 2.

Primary cultures of endometrial cells

Epithelial and stromal cells were isolated from bovine endometrium collected from mixed breed beef cows on day 11–17 of the oestrous cycle, as previously described (Cronin *et al.* 2012). The cells were treated with control medium (RPMI-1640 medium (Sigma–Aldrich), 10% heat-inactivated foetal bovine serum (Sigma–Aldrich), 1% penicillin–streptomycin (Sigma–Aldrich), 1% amphotericin B (Sigma–Aldrich)) or a medium containing roIFNT (100 ng/ml) for 2 h (Mansouri-Attia *et al.* 2009a). Each experiment was carried out using isolated cells from four independent animals.

Immunohistochemistry

The endometrial samples were fixed in 4% paraformaldehyde in PBS, as previously described (Eozenou *et al.* 2012). After three washes with PBS, the samples were dehydrated by treating with increasing concentrations of ethanol (30%, 50%, 70%, 90%, 100%) in a Shandon Citadel 1000 tissue processor (Thermo Scientific, Courtaboeuf, France) for 12 h. The tissues were embedded in paraffin and stored at 4°C until further processing.

The sections (7 μm) were cut with a microtome Leica RM2245 (Leica, Nanterre, France) for immunohistochemistry as previously described (Eozenou *et al.* 2012). The slides were incubated with primary antibodies, anti-SOCS3 (dilution 1:200, sc9323, Santa Cruz Biotechnology) and anti-SOCS6 (dilution 1:200, ab53181, Abcam, Paris, France), in phosphate buffer (0.1 M, pH 7.4, with 2% BSA (Sigma–Aldrich) and 1% normal donkey serum (Millipore, Guyancourt, France)) at 4°C overnight. As negative controls, the sections were incubated in phosphate buffer without primary antibody. The slides were incubated with an anti-rabbit secondary antibody (dilution 1:40; sc-346, Santa Cruz Biotechnology) for 1 h at room temperature and then washed three times and incubated with diaminobenzidine substrate and urea (Sigmafast 3,3'-diaminobenzidine tablets, Sigma–Aldrich) in Tris-buffer for 6–8 min until colour developed. After one wash in water, the slides were dehydrated through ascending concentrations of ethanol and then mounted with Eukitt mounting medium (Sigma–Aldrich). A section from each physiological condition was stained with haematoxylin–eosin solution to highlight tissue morphology. The images were obtained using a NanoZoomer digital pathology system and analysed using a NanoZoomer digital pathology virtual slide viewer (NDPView software, Hamamatsu, Japan). Each experiment was repeated for four females per biological condition. Four fields per animal were analysed. To evaluate protein expression, three independent observers analysed obtained images and determined protein intensity in each sub-cellular population of endometrium.

Total RNA extraction

Total RNA was extracted from frozen tissue by homogenisation in Trizol Reagent (Invitrogen) according to the manufacturer's recommendations and as previously published (Mansouri-Attia *et al.* 2009a, Eozenou *et al.* 2012). Total RNA samples were purified on Qiagen columns according to the manufacturer's protocol (RNeasy Mini Kit, Qiagen). The cultured primary cells were washed with 1 ml of PBS and then total RNA was extracted using the RNeasy Mini Kit and the automated system Qiacube (Qiagen; Cronin *et al.* 2012). Quality and integrity of extracted and purified total RNA were determined using an Agilent 2100 bioanalyser (Agilent, Les Ulis, France). 1 µl of RNase inhibitor (RNasin, Promega) was added to each sample before storing at -80 °C.

Quantitative real-time PCR

As previously described (Mansouri-Attia *et al.* 2009a, Eozenou *et al.* 2012), 1 µg of total RNA was reverse-transcribed into cDNA using OligodT and SuperScript II (Invitrogen). Quantitative real-time PCR (qPCR) was carried out with Master Mix SYBR Green (Applied Biosystems) and Step One Plus system (Applied Biosystems). The primers were designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) or Primer Express Software (Applied Biosystems) and then synthesised by Eurogentec (Angers, France). The oligonucleotide primers used for gene quantification are listed in Table 1. To assess the amplification of the correct cDNA fragments, every amplicon was sequenced and blasted on NCBI RNA bovine collection. For each gene of interest, relative expression was normalised to the expression of the most stable reference genes as determined by qBaseplus software from the quantification of six housekeeping genes (Biogazelle, Ghent, Belgium).

Statistical analyses

All statistical analyses were performed with GraphPad Prism 6 software (La Jolla, CA, USA). *SOCS* gene expression in tissues was first subjected to a two-way ANOVA followed by Student's *t*-test tests to analyse the effect of day, pregnancy status (cyclic or pregnant), endometrial areas (CAR and ICAR), and their

interactions. *SOCS* expression in primary cell cultures treated by rolFNT was subjected to a two-way ANOVA analysis followed by Student's *t*-test tests to study the potential differential *SOCS* expression relative to cell lines and to rolFNT treatment.

Results

Endometrial *SOCS* expression during the oestrous cycle and pre-implantation period

Using bovine endometrium collected from cross-bred beef heifers, *SOCS* mRNA levels were analysed in the CAR and ICAR endometrial areas at day 16 and day 20 of the oestrous cycle and pregnancy. In cycling heifers, the presence of a regressed or a P₄-secreting functional corpus luteum was confirmed by the transcriptional level of *FOXL2*, a transcription factor whose endometrial mRNA expression is inversely correlated with P₄ circulating level (Eozenou *et al.* 2012).

From day 16 to day 20 post-oestrus (Fig. 1), no significant difference in *CISH*, *SOCS4* and *SOCS7* mRNA levels was observed in the endometrium sampled from cyclic heifers, whereas *SOCS3* mRNA level increased in CAR and ICAR areas; *SOCS5* and *SOCS6* gene expression decreased in both endometrial areas and *SOCS1* and *SOCS2* gene expression increased in ICAR areas only.

In the pregnant endometrium (Fig. 1), no significant variation in *CISH*, *SOCS2*, *SOCS5* and *SOCS7* mRNA expression was seen in either endometrial area between the stage of maternal pregnancy recognition (day 16) and implantation (day 20). At the same period, a significant increase was detected for *SOCS4* transcript in the CAR areas and for *SOCS1*, *SOCS3* and *SOCS6* transcripts in CAR and ICAR areas.

When pregnant endometrium was compared with cyclic endometrium (Fig. 1 and Table 2), distinct patterns of *SOCS* mRNA regulation were observed, ranging from the absence of significant change (*CISH*, *SOCS7*) to a significant up-regulation of mRNA level in CAR and ICAR areas at day 16 and day 20 (*SOCS6*).

Table 1 Description of the oligonucleotide primers used for bovine gene quantification by real-time RT-PCR.

Gene	Forward	Reverse	Amplicon size	Accession number
<i>CISH</i>	AGTCATCCTGGTGCCCGTGA	AGCCTTGTTCTCTGCACACCG	69	NM_001046586
<i>SOCS1</i>	CTCGTACCTCTACCTCTTCATGTT	ACAGCAGAAAAATAAGCCAGAGA	92	XM_864316
<i>SOCS2</i>	TGCAAGGATAAGCGGACAGG	AGATGCTGCAGAGGTGGTGCT	101	NM_177523
<i>SOCS3</i>	GCCACTCTCCAACATCTCTGT	TCCAGGAACCTCCGAATGG	97	NM_174466
<i>SOCS4</i>	GCTTTGTAACTATGTCATTGGCA	ACAACACACACAGCTTTACCGAAG	100	NM_001076218
<i>SOCS5</i>	TACATCCAGTGGCTGTGCG	GCACAGCAAGCAGAAACATACATT	100	NM_001046182
<i>SOCS6</i>	AGAAGGTCAAAAAATGTCACAGGAA	CAATGGTTTATGCTTTTGGCAT	113	XM_005224062
<i>SOCS7</i>	CTCCCACTGCCTAAGCCTCTG	GAAATGAGCTGCGCTTCCTT	102	NM_001206013
<i>ACTB</i>	CCTGGCACCCAGCACAA	AGCGAGGCCAGGATGGA	90	NM_173979
<i>CNOT11 (C2ORF29)</i>	CTTCAAGAGCCCCCTGT	GGGTCCTTTTCCAACCTCC	64	XM_002691150
<i>GAPDH</i>	GCTGACGCTCCCATGTTTGT	TCATAAGTCCCTCCACGATGC	432	NM_001034034
<i>RPL19</i>	CCCCAATGAGACCAATGAAATC	CAGCCCATCTTTGATCAGCTT	72	NM_001040516
<i>SLC30A6</i>	TGATGAGGAAACCTAGCCCTGCC	TCGGGCTGCTCAAAAAGCGT	142	NM_001075766
<i>SUZ12</i>	CGTTGTGAGCAGTTTGCCCTGT	ACCACAGTCTGGAGTTGGACT	139	NM_001205587

Figure 1 Quantification of *SOCS* gene expression in cyclic and pregnant bovine endometrium on day 16 and 20 of the oestrous/pregnancy. Caruncular (CAR) and intercaruncular (ICAR) areas were collected from cyclic (day 16: $n=5$; day 20, $n=6$) and pregnant (day 16: $n=4$; day 20: $n=5$) cross-bred heifers. Expression of *SOCS* was normalised to that of *RPL19* and *SCL30A6* using qBasePlus. Scale Bars (mean $\pm$ S.E.M.) with different lowercase letters differ significantly ($P < 0.05$ or lower).

Regulation of endometrial SOCS expression by IFNT

In order to determine the short-term impact of IFNT on *SOCS* gene expression, Charolais cows were infused with rolFNT for 2 h at day 14 of the oestrous cycle (Fig. 2). The primary cultures of endometrial cells (epithelial or stromal origin) were also derived and incubated with rolFNT for 2 h (Fig. 3). In these experimental models, no impact of IFNT on *SOCS4*, *SOCS5*, *SOCS6* and *SOCS7* transcript levels was seen *in vivo* or *in vitro* (Figs 2 and 3).

Intrauterine infusions with rolFNT significantly increased the expression of *SOCS1* (4.4-fold in CAR; 6.8-fold in ICAR), *SOCS2* (1.8-fold in CAR; 2.8-fold in ICAR) and *SOCS3* (fourfold in CAR) mRNA levels (Fig. 2). The expression of *CISH* mRNA was also up-regulated by rolFNT treatment when CAR and ICAR endometrial areas were considered together.

In primary cultures of endometrial cells (Fig. 3), *CISH* and *SOCS1* mRNA levels were significantly up-regulated by rolFNT in stromal cells (three- and 8.3-fold respectively), whereas *SOCS1* and *SOCS3* mRNA levels were significantly up-regulated by rolFNT in epithelial cells (ten- and 2.3-fold respectively). No significant regulation of *SOCS2* transcript expression by IFNT was observed in either cell types. Interestingly, mRNA expression levels of *CISH* and *SOCS4* were lower and those of *SOCS1* and *SOCS2* were higher in the primary cultures of stromal cells compared with epithelial cells (Fig. 3).

Regulation of endometrial SOCS expression by exogenous P_4

In order to investigate the impact of P_4 on *SOCS* mRNA expression, cross-bred beef heifers were supplemented with P_4 from day 3 of the oestrous cycle onwards and the endometrium was sampled at day 5 or day 16 (Fig. 4). A significant increase in mRNA levels was seen for *SOCS6* ($P < 0.05$) and *SOCS7* ($P < 0.01$) in the P_4 -treated group (day 5 and day 16) compared with the group of control heifers. *SOCS5* gene expression was significantly up-regulated by 13 days of P_4 treatment (2.8-fold, $P < 0.01$). No significant impact of P_4 was observed for *CISH*, *SOCS1* and *SOCS2* transcripts. Interestingly, for *CISH* only, this experiment showed an increased expression in endometrial mRNA level from day 5 to day 16 of the oestrous cycle (Fig. 4; $P < 0.05$). No significant correlation was found between P_4 blood concentration and any of the *SOCS* transcript expression in the endometrium (data not shown).

Immunolocalisation of *SOCS3* and *SOCS6* in bovine endometrium

SOCS3 and *SOCS6* were localised in endometrial tissues sampled from cyclic Charolais cows on day 14 (luteal phase) and day 20 (follicular phase) as well as in pregnant cows at the time of initiation of implantation (Fig. 5, day 20). During the oestrous cycle, *SOCS3* and *SOCS6* were detected in luminal, stromal and glandular cells.

Table 2 Effect of pregnancy on bovine *SOCS* genes expression (fold changes) in caruncular (CAR) and intercaruncular (ICAR) areas at day 16 (D16) and day 20 (D20) after oestrus.

	CAR		ICAR	
	D16	D20	D16	D20
<i>CISH</i>	0.96	0.99	0.83	1.04
<i>SOCS1</i>	0.93	1.48 [†]	1.38*	1.16
<i>SOCS2</i>	1.11	1.09	1.16	0.56 [†]
<i>SOCS3</i>	1.45*	2.17 [§]	1.85*	1.44
<i>SOCS4</i>	1.10	1.26 [†]	0.99	0.97
<i>SOCS5</i>	0.99	1.54 [†]	0.91	1.57 [†]
<i>SOCS6</i>	1.25 [†]	3.47 [§]	1.33 [†]	2.74 [§]
<i>SOCS7</i>	1.01	0.97	1.26	1.03

* $P < 0.05$; [†] $P < 0.01$; [‡] $P < 0.001$; [§] $P < 0.0001$.

Figure 2 Regulation of endometrial SOCS gene expression by IFNT *in vivo*. Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from Charolais cows infused with control solution ($n=5$) or roIFNT (200 $\mu\text{g}/\text{ml}$; $n=5$) for 2 h at day 14 of oestrous cycle. SOCS mRNA levels were quantified by RT-qPCR and normalised to *CNOT11*, *SLC30A6* and *SUZ12* using qBasePlus. Data are the mean $\pm$ S.E.M. x and y letters indicate significant differences in total endometrial expression between control and IFNT samples ($P<0.05$). Asterisks denote significant effects ($*P<0.05$).

Compared with cyclic endometrium (day 14 or day 20), nuclear SOCS3 staining in pregnant endometrium appeared with the same intensity in stromal cells and luminal epithelium, but more intense in glandular cells (Table 3). In luminal, glandular and stromal cells, nuclear SOCS6 staining was less intense in the late-luteal phase (day 20) compared with the mid-luteal phase (day 14).

In luminal cells, SOCS6 staining intensity increased at day 20 of pregnancy (when implantation takes place) compared with either day of the oestrous cycle.

Regulation of endometrial SOCS genes by SCNT concepti

The endometrium has been shown to distinguish concepti displaying distinct potencies of development

Figure 3 Regulation of SOCS gene expression by IFNT in bovine endometrial cells. *In vitro*, cells isolated from bovine endometrium were treated with roIFNT (100 ng/ml) for 2 h. For each gene, mRNA expression was normalised to that of *RPL19* and *ACTB* using qBasePlus. Data are the mean $\pm$ S.E.M. Asterisks denote significant effects ($*P<0.05$; $**P<0.01$; $***P<0.001$; $****P<0.0001$).

Figure 4 SOCS endometrial gene expression in cyclic and pregnant cross-bred beef heifers supplemented with P_4 from day 3 after oestrus. The females were slaughtered after 2 days (day 5 after oestrus, normal P_4 level in cyclic heifers, $n=5$ and high P_4 level in cyclic heifers, $n=5$) and 13 days (day 16 after oestrus, normal P_4 level in cyclic heifers, $n=4$ and high P_4 level in cyclic heifers, $n=5$) of P_4 supplementation. SOCS mRNA level was quantified by RT-qPCR and normalised to *ACTB* and *RPL19* using qBasePlus. Scale Bars (mean $\pm$ S.E.M.) represent the different conditions. Asterisks denote significant effects (* $P<0.05$, ** $P<0.01$) x and y letters indicate significant difference between day 5 and day 16 samples ($P<0.05$).

to term (Mansouri-Attia *et al.* 2009b). We investigated the expression of endometrial SOCS genes in day 20 pregnant heifers carrying SCNT concepti compared with control pregnancies. SOCS3 mRNA was significantly increased in the endometrium of SCNT pregnancies

(Fig. 6; $P<0.05$) whereas other SOCS genes did not present any significant variation in mRNA concentration.

Discussion

The family of SOCS proteins has been shown to play essential functions in the negative regulation of cell signalling pathways activated by cytokine and hormone receptors, most of them being expressed at the embryo–maternal interface in mammals (Krebs & Hilton 2001, Fitzgerald *et al.* 2009). The endometrium is critical for supporting implantation and this depends on its physiological status as well as the developmental potency and quality of the embryo with which it interacts (Salamonsen *et al.* 2009, Sandra *et al.* 2011, Weimar

Figure 5 Cellular localisation of SOCS3 and SOCS6 protein in cyclic or pregnant bovine endometrium. Immunohistochemistry was carried out using sections cut from Charolais cows sampled at day 14 (14Cy; A, D, G, J, M, and P) and day 20 of the oestrous cycle (20Cy; B, E, H, K, N, and Q) as well as day 20 (20Pr; C, F, I, L, O, and R) of pregnancy. A–I and J–R sections were respectively incubated with anti-SOCS3 and anti-SOCS6 primary antibody. Tissue samples in S were incubated without primary antibody as a negative control. Tissue samples in T–U were stained with haematoxylin–eosin solution. BV, blood vessel; GE, glandular epithelium; LE, luminal epithelium; S, stroma. Scale bars = 100 μ m.

Table 3 Nuclear distribution of SOCS3 and SOCS6 proteins expression in bovine endometrium at day 14 (14Cy) or day 20 (20Cy) of cycle and day 20 (20Pr) of pregnancy.

	Luminal epithelium	Stroma	Glands
SOCS3			
14Cy	+	++	++
20Cy	-/+	++	++
20Pr	+/++	+/++++	++++
SOCS6			
14Cy	++	+++	+++
20Cy	+	++	++
20Pr	+++	+++	+++

–, no staining; +, weak staining; ++, pronounced staining; +++, intense staining; +++++, very intense staining.

et al. 2012). Although the contribution/function of various endometrial factors (ligands, receptors and effectors) in the control of implantation has been unveiled based on descriptive and functional studies, few publications have focused on the regulation of *SOCS* genes and their biological functions in the uterus (Sandra *et al.* 2005, Oliveira *et al.* 2012). In order to provide new insights into the involvement of *SOCS* in endometrial physiology, a comprehensive analysis of known *SOCS* (*CISH*, *SOCS1*–7) gene expression profiles was carried out in bovine endometrium in order to i) establish mRNA expression patterns of the *SOCS* gene family during the oestrous cycle and early pregnancy, ii) determine the cellular localisation of *SOCS3* and *SOCS6* protein during the oestrous cycle and early pregnancy, iii) define the contribution of IFNT and of P_4 in the regulation of the *SOCS* genes and iv) determine if endometrial *SOCS* mRNA levels are altered in a model of compromised pregnancy using bovine SCNT concepti.

In the bovine endometrium, all *SOCS* genes examined were shown to be expressed from day 5 to 20 of the oestrous cycle (present study and N Forde, A Vitorino Carvalho 2013, unpublished observations). The comparison of a late-luteal time point (day 16) with the period of luteolysis (proestrus: day 20) revealed higher mRNA concentrations for *SOCS1*, *SOCS2* and *SOCS3* genes but lower concentrations of *SOCS5* and *SOCS6* at the latter time point (day 20). A noteworthy rise in *CISH* gene transcription could also be seen between the early and late stages of the luteal phase (day 5 vs day 16 of the oestrous cycle), which was not followed by a decline at the luteolytic stage (day 20). In previously published work based on dot-blotting analyses, no change in endometrial expression of ovine *CISH*, *SOCS1*, *SOCS2* and *SOCS3* mRNA levels was reported during the oestrous cycle (Sandra *et al.* 2005). This likely reflects the lower sensitivity of the dot-blotting technique compared with the real-time PCR analyses undertaken in this study. Interestingly, microarray analysis of human endometrium (Talbi *et al.* 2006) has shown variations in *CISH*, *SOCS1*, *SOCS2*, *SOCS3* and *SOCS6* gene levels in the secretory phase compared with the proliferative

phase. In total, these findings from both human and cattle studies indicate that endometrial *SOCS1*, *SOCS2*, *SOCS3* and *SOCS6* gene expression is sensitive to ovarian steroids indicative of a regulatory role. Modulation of *SOCS* gene expression by oestrogen or P_4 has already been documented in hepatic tissues and cultured

Figure 6 Endometrial *SOCS* gene expression in Holstein heifers carrying embryos produced by artificial insemination or transferred with SCNT produced embryos. Four females were slaughtered for each type of pregnancy at day 20 post-oestrus. Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected separately. *SOCS* mRNA levels were quantified by RT-qPCR and normalised by qBasePlus on *CNOT11*, *RPL19* and *SLC30A6*. Scale bars are representative of the mean value $\pm$ S.E.M. x and y letters indicate significant difference between control and SCNT pregnancy ($P < 0.05$).

cells of breast or neuronal origin (Leong *et al.* 2004, Matthews *et al.* 2005, Steyn *et al.* 2008). P₄-regulated SOCS factors could therefore be involved in the cross regulation of signalling pathways implicated in endometrium remodelling that takes place at the end of the oestrous cycle.

Using an *in vivo* experimental model of P₄-supplemented heifers, we showed that P₄ treatment i) led to the significant up-regulation of SOCS5, SOCS6 and SOCS7 transcript expression consistent with the decline observed at day 20 of the oestrous cycle when P₄ blood level drops, and ii) did not affect CISH, SOCS1, SOCS2 and SOCS3 transcript expression. Since in non-supplemented cycles, endometrial SOCS1, SOCS2 and SOCS3 transcript levels do increase from day 16 to day 20 when luteal regression occurs, it is likely that regulatory factors other than P₄ are involved. In the endometrium, during luteolysis, various parameters could account for the increased expression of these SOCS genes, including modifications of the cytokines/hormones balance (Rahman *et al.* 2004) and variations in type and/or number of immune cell populations such as macrophages and dendritic cells that take place in the endometrium (Cobb & Watson 1995, Mansouri-Attia *et al.* 2012, Oliveira *et al.* 2012). Whereas the increase in CISH mRNA expression appears to be related to the acquisition of endometrial receptivity, expression changes in other SOCS genes during the oestrous cycle coincide with luteolysis and reflect a direct or an indirect consequence of declining P₄ blood levels, with the identity of intermediary factors remaining to be uncovered.

In the pregnant CAR or ICAR endometrial areas compared with the cyclic tissues, distinct patterns of SOCS transcript regulation were observed. Indeed, for SOCS6, the mRNA level first rose during the maternal phase of pregnancy recognition (day 16 of pregnancy) and was further amplified at implantation (day 20 of pregnancy). Moreover, SOCS1 and SOCS3 transcript levels rose specifically at the time of implantation. This regulation of endometrial SOCS mRNA expression by the conceptus is consistent with microarray studies that listed several differentially expressed SOCS genes in the bovine pregnant endometrium compared with cyclic tissue collected at day 17 (SOCS1, Walker *et al.* 2010), day 18 (SOCS1, SOCS3, SOCS4, SOCS6, Bauersachs *et al.* 2012) and day 20 (SOCS6, Mansouri-Attia *et al.* 2009a) after oestrus. Interestingly, the expression patterns of CISH, SOCS1, SOCS2 and SOCS3 mRNA reported in this study are similar to the changes observed in the ovine endometrium (Sandra *et al.* 2005). In this species, endometrial CISH, SOCS1, SOCS2 and SOCS3 mRNA levels did not vary in ovine CAR areas during the period of maternal recognition (day 12 of pregnancy) compared with day 12 of oestrous cycle (luteal phase), but they clearly increased at day 16 of pregnancy when implantation is initiated. In cattle, at day 16 of pregnancy (maternal pregnancy recognition) compared with day 16

of the oestrous cycle (luteal phase), endometrial SOCS1, SOCS2 and SOCS3 mRNA levels were similar. However, the transcript levels of all three genes were significantly upregulated at day 20 when apposition of the extra-embryonic tissues on the endometrium occurs. Altogether, global transcriptional profiles of endometrial CISH, SOCS1, SOCS2 and SOCS3 genes do not suggest critical functions for these factors during the maternal period of pregnancy recognition, but they do indicate a possible biological role for SOCS genes in driving the process of implantation in ruminants.

Although the regulation of several SOCS genes depends upon IFNT at days 16 and 20 of pregnancy, mRNA expression profiles as well as the cell localisation studies described in this study indicate that other factors participate in the regulation of endometrial SOCS gene expression. SOCS3 protein was detected in the cytoplasm and the nucleus of major cell populations that compose the endometrium including luminal epithelium, stromal cells and glandular epithelium. At implantation, when IFNT secretion declines, staining for SOCS3 was more intense in the nucleus of glandular cells, a subcellular localisation previously reported when SOCS3 is expressed at a high level in cells (Lee *et al.* 2008). These glandular cells secrete histotroph whose production is essential for the development and growth of the conceptus (Gray *et al.* 2001). In ruminants, the regulation of endometrial gland function involves P₄, IFNT as well as various peptidic hormones and factors including placental lactogens and the growth hormone/insulin growth factor (GH/IGF) family (Wathes *et al.* 1998, Spencer *et al.* 2004). During the pre-implantation period, IGF1 and IGF2 levels have been shown to increase in ovine and bovine endometrium (Geisert *et al.* 1991, Kirby *et al.* 1996, Keller *et al.* 1998, McCarthy *et al.* 2012) and IGF components are principally expressed in luminal and glandular epithelia of the bovine endometrium (McCarthy *et al.* 2012). SOCS proteins have been shown to modulate the JAK-STAT pathway activated by IGF and GH (Krebs & Hilton 2001, Fujimoto & Naka 2003). Placental lactogen (PL or chorionic somatomammotropin hormone (CSH)), a member of the prolactin (PRL)/GH family, is secreted by the bovine binucleate trophoblast cells from day 18 of pregnancy onwards (Kessler *et al.* 1991, Wooding *et al.* 1992) and, as for ovine CSH, its biological actions require PRL and GH receptors whose biological actions are mediated by the JAK-STAT-SOCS pathways (Biener *et al.* 2003, Spencer *et al.* 2004). Thus, from day 20 of pregnancy onwards, the increased SOCS3 in the endometrial glands could reflect the contribution of this SOCS factor in the negative control of signalling pathways stimulated by CSH and IGF ligands.

A major and unique finding of this study is that endometrial SOCS6 expression undergoes a clear increase during the maternal recognition phase and implantation. Our data demonstrate that SOCS6 is not an

IFNT-induced nor regulated gene *in vivo*, confirmed by the inability of IFNT to affect *SOCS6* mRNA expression at 2 or 24 h *in vitro* (this study and Vitorino Carvalho *et al.* (2014)). Interestingly, as for *SOCS3*, *SOCS6* protein was detected in the cytoplasm and the nucleus of luminal epithelium, stroma cells and glandular epithelium of the endometrium. The nuclear staining of *SOCS6* is consistent with published data indicating that a high level of *SOCS6* expression leads to its nuclear localisation in cells (Hwang *et al.* 2007). Although *SOCS6* regulators and its biological functions have been less investigated than for other *SOCS* genes, two relevant clues have recently been provided by reports showing that *SOCS6* can i) potentiate signalling of the IFN type I receptor by targeting *SOCS1* to induce *SOCS1* proteosomal turnover (Piessevaux *et al.* 2006) and ii) downregulate *STAT3* protein levels in HEK293T cells (Hwang *et al.* 2007). *STAT3* is expressed in the endometrium of ruminants (Song *et al.* 2009) and the mRNA expression profiles of the *SOCS6* and *SOCS1* genes are similar in pregnant females. This suggests that *SOCS6* plays a role in the regulation of *STAT3* and *SOCS1* availability in the bovine endometrium. Additional studies based on the alteration of *SOCS* gene expression in primary cultures of endometrial cells could help in deciphering the biological roles of *SOCS6* in the endometrium.

Biosensing of embryo quality is an endometrial property that has been established in cattle (Mansouri-Attia *et al.* 2009b) and humans (Teklenburg *et al.* 2010). As previously reported by microarray analyses (Mansouri-Attia *et al.* 2009b), SCNT concepti elicit an endometrial response that, in turn, affects the progress of implantation and is associated with pregnancy failures. This study indicates an increased expression of *SOCS3* mRNA levels in the endometrium of SCNT concepti-carrying heifers. In transgenic mice, *Socs3* overexpression leads to an embryonic lethality (as a consequence of anaemia) and to increased TH2 responses (reviewed in Fitzgerald *et al.* (2009)). This later phenotype suggests a link between *SOCS3* and the deregulated immune function we have previously reported in the endometrium of SCNT pregnancies (Mansouri-Attia *et al.* 2009b). This increase in endometrial *SOCS3* likely reflects an alteration in the quality or the type of embryonic signals emanating from SCNT concepti. Using day 18 SCNT concepti, derived from the 5538 somatic cell line (the same as the one used in our work), recent transcriptomic analyses have not focused on ligands or secreted factors that could differ with AI or *in vitro* produced (IVP) concepti (Degrelle *et al.* 2012). Nevertheless, these analyses did show that bovine *IFNT* as well as *CSH* gene expressions are identical between AI concepti and SCNT concepti derived from the 5538 cell line (Degrelle *et al.* 2012, Mansouri-Attia *et al.* 2009b; Vitorino Carvalho A, unpublished observations). Based on recent data reporting the biological functions

of embryo signals other than IFNT, glucocorticoids and PGs produced by the trophectoderm and the endometrium are also part of the orchestra that regulates the expression of endometrial genes during implantation in ruminants (Dorniak *et al.* 2011, Majewska *et al.* 2012, Ulbrich *et al.* 2012). Compared with pregnancies obtained by IVP embryos, uterine flushings from bovine SCNT pregnancies were reported to contain lower levels of PGI2 and PGE2 due to a reduced amount of embryo-and/or endometrium produced PG (reviewed by Ulbrich *et al.* (2012)). In rat corpus luteum and hypothalamus, PG has been shown to regulate *SOCS1* and *SOCS3* expression (Curlewis *et al.* 2002, Bonafede *et al.* 2011). Altogether these data suggest that the higher level of endometrial *SOCS3* in SCNT pregnancy could reflect an altered production and/or metabolism of PG. In this model of compromised implantation with long-term effects, the better understanding of the events taking place in the endometrium facing cloned embryos requires a thorough characterisation of the extra-cellular signals that differ between cloned and control embryos.

Given the fact that *SOCS* factors not only act in a negative feedback loop to inhibit signal transduction, but also have cross-talking functions which regulate other signalling pathways, disentangling the contribution of the *SOCS* gene family in the regulation of endometrial function represents a challenge. Nevertheless our *in vivo* and *in vitro* models have demonstrated rapid changes in endometrial expression of *SOCS3* and *SOCS6* as well as *SOCS1* and *CISH* gene levels during cycle and early pregnancy as well as in response to two major factors of the pregnancy, IFNT and P₄, in cattle. Consequently, appropriate spatio-temporal expression of *SOCS* genes is likely necessary to fine-tune endometrial physiology during the acquisition of receptivity, maternal recognition of pregnancy and implantation. Alterations in endometrial *SOCS* gene expression may be detrimental for the progression of any of these steps therefore affecting global reproductive capacity of the female. Intensive efforts are currently underway to identify biomarkers of endometrial quality as well as targets for therapeutic approaches with the aim of preventing embryo implantation (contraception) or to restore the ability of the endometrium to support term pregnancy. In this context, the role of *SOCS* genes in the mammalian endometrium deserves further attention, particularly when functional capacity is compromised in response to environmental insults such as nutrition, stress or infections.

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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