ISGylation: A Conserved Pathway in Mammalian Pregnancy

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Abstract

Successful pregnancy includes remodeling and differentiation of the endometrium in response to sex steroid hormones, development of maternal immunotolerance to the implanting embryo, and modification of the local uterine environment by the embryo to suit its own needs. The major signal released by the ruminant conceptus during establishment of pregnancy is interferon-tau (IFNT) that stimulates the expression of many genes in the endometrium and ovary. One of these genes is called interferon stimulated gene 15 (ISG15), which encodes a ubiquitin homolog with a C-terminal Gly that becomes covalently attached to Lys residues on targeted proteins through an ATP-dependent multi-step enzymatic reaction called ISGylation. The conceptus-derived induction of endometrial ISGs also occurs in mouse and human deciduas and placenta, in response to pregnancy presumably through action of cytokines such as interleukins and type I IFN. Described herein is evidence to support the concept that ISGylation is a maternal response to the developing conceptus, implantation and placentation that is conserved across mammalian pregnancy. Although the precise role for ISG15 remains elusive during pregnancy, it is clear that up-regulation in response to pregnancy may impart a pre-emptive defense to infection or other environmental insults, and protection of the conceptus against inflammatory insults across species.

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P. Sutovsky (ed.), *Posttranslational Protein Modifications in the Reproductive System*, Advances in Experimental Medicine and Biology 759, DOI 10.1007/978-1-4939-0817-2_2,

Keywords
Conceptus • Decidua • Endometrium • Evolutionary conservation • Interferon • ISG15 • Pregnancy • Ubiquitin

Introduction

The most abundant protein secreted from cultured ovine conceptuses was isolated during the peri-implantation period [1]. Through adding radiolabeled leucine in leucine-deficient medium, this conceptus-derived secretory product was demonstrated to be released by using a 2D-PAGE approach [2]. Large-scale purification of this acidic protein, subsequently called trophoblast protein 1 or TP-1, led to studies demonstrating that it bound to endometrial receptors [2, 3], induced upregulation of many endometrial proteins [2, 4–7], altered release of luteolytic prostaglandin $F_2\alpha$ (PGF) [8, 9] and resulted in extended estrous cycles when infused into the uterine lumen of cyclic ewes. The inferred amino acid sequence from cloned cDNAs revealed homology with ovine [10] and bovine [11] type I IFN cDNAs. TP-1 was subsequently found to be encoded by several genes [12, 13] and was later renamed IFNT.

The general IFN response, characterized by increased expression of ISGs observed in ruminant species during early pregnancy, is conserved across species with different modes of implantation. Mice and humans lack the IFNT gene; however, embryo-derived factors other than IFNT are known to activate an IFN-like response. For example, many other Type I IFN (α, β, δ, ε, ω) and cytokines such as interleukins also activate JAK/STAT signal transduction and increase expression of ISGs. In humans, there is strong evidence for induction of ISGs in endometrial decidual cells when cultured with conditioned human trophoblast cell medium [14]. One candidate for induction of ISGs in mice and in humans might be interleukins [15]. This chapter describes the identification of ISG15 as a maternal response to pregnancy in many animal models including humans. It also presents data to support a functional role for ISGylation and post-translational modification of proteins based on negative impact of gene deletion on both establishment of pregnancy and liter size in mice.

IFNT Functions as an Anti-Luteolytic Signal

PGF is released from the luminal epithelium of the endometrium and is the luteolytic factor in ruminants [16–18]. Oxytocin enhances cyclooxygenase-2 (*COX2*) transcription, which increases pulse amplitude of PGF production by the endometrium [19]. In turn, PGF promotes both functional (i.e., decline in progesterone biosynthesis) and structural (i.e., programmed cell death) regression of the corpus luteum, thereby initiating a new estrous cycle. Maternal recognition of pregnancy in ruminant species is successful only when PGF synthesis and release from the endometrium is attenuated. Luteolysis is averted during pregnancy by conceptus secreted IFNT.

Early pregnancy is maintained in ruminants through the actions of conceptus-derived IFNT on the endometrium. IFNT alters uterine release of PGF, which

results in rescue of the corpus luteum and continued secretion of progesterone. In sheep, cumulative PGF is not altered during early pregnancy [20]. Rather, IFNT suppresses up-regulation of estrogen receptor gene expression [21], which is in turn necessary for up-regulation of oxytocin receptor (*OTR*) in the absence of pregnancy [22]. At least in the ewe, IFNT exerts its inhibitory actions, at the proximal end of the signaling pathway mediating PGF secretion (i.e., OTR). This may help explain how IFNT disrupts pulsatile rather than total PGF release in the ewe. Unlike the promoter for the *OTR* gene of the ewe, the bovine *OTR* gene lacks a classical palindromic estrogen response element [23] and no change in ESR1 expression has been observed [24]. Using in situ hybridization, Robinson et al. showed that despite the slight decrease in OTR mRNA in endometrium from Day 16 pregnant cows when compared with estrous cycling cows, estrogen receptor mRNA does not change [24]. Thus, pregnancy can apparently alter OTR mRNA expression exclusive of the estrogen receptor in cows. Because IFNT acts in paracrine fashion directly on the endometrium, several groups have sought to establish transcriptional responses to IFNT exposure in the endometrium [25, 26], as well as endocrine responses in the ovary [25, 27–29]. One subset of genes that become upregulated in response to IFNT in reproductive tissues are the interferon stimulated genes (ISGs).

Interferon-Stimulated Genes: Identification of ISG15 in the Bovine Uterus

Studies were initiated on endometrial response to pregnancy and conceptus-derived IFNT in the early 1990s. In 1995, a ~16 kDa protein was described that was released into media by endometrial explants cultured from day 18 pregnant cows and endometrial explants from non-pregnant cows that were treated in vitro with recombinant bovine IFNT (rboIFNT) [5]. A protein of similar size, ~14.4 kDa, was previously described following culture of Ehrlich ascites tumor cells with other type 1 IFN [30]. About five years later, the same protein was described in human fibroblasts and MDBK cells when treated with IFNA or IFNB [31]. The function and identity of this ISG remained unknown until Dr. Haas examined regulation of intracellular ubiquitinated proteins in response to viral infection [32]. When completing western blots of intracellular proteins with antibody against ubiquitin following viral infection, a 15 kDa protein was up-regulated that immunoreacted with antibody against ubiquitin. This apparent 15 kDa protein, subsequently named ubiquitin cross-reactive protein (UCRP), maintained amino acid sequence identity with a tandem ubiquitin repeat and was upregulated following exposure to IFNA and IFNB, and to a lesser extent following culture with IFNG. By using affinity-purified antibody against UCRP that did not cross-react with ubiquitin, these investigators described conjugation of UCRP to proteins in response to IFN [33] which utilized distinct activating and conjugating enzymes, and resulted in UCRP—protein conjugates that were distinct from those conjugated to ubiquitin [34].

By using anti-ubiquitin antibody and anti-UCRP antibody from Dr. A.L. Haas, the ~16 kDa endometrial protein shown to be upregulated and secreted in response

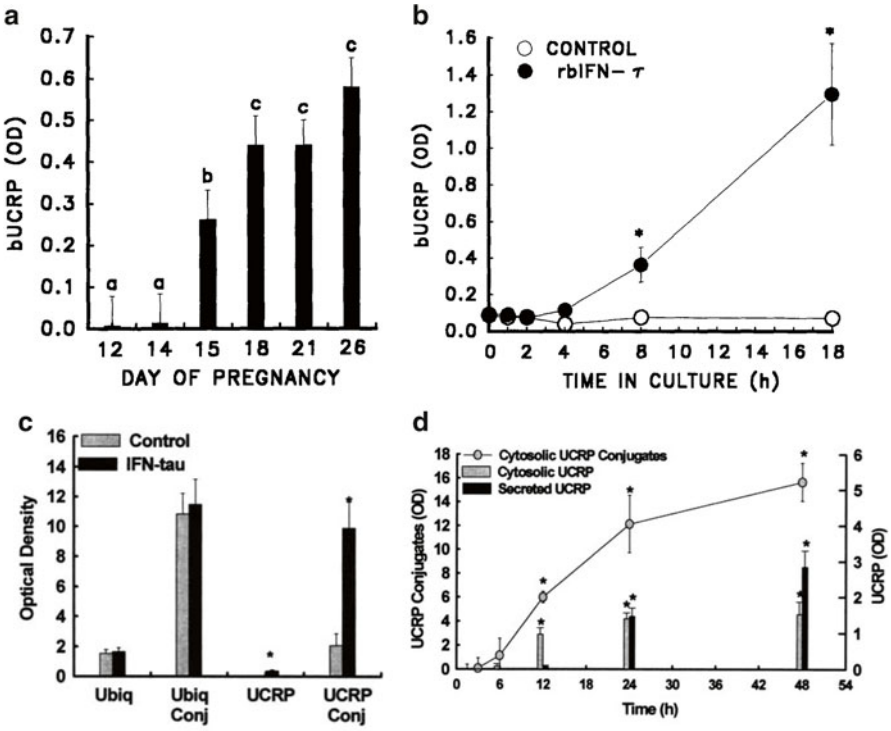


Fig. 2.1 Temporal endometrial release and intracellular endometrial concentrations of UCRP in response to pregnancy and time in culture in response to IFNT treatment. Panel **a** describes release of ^{3}H-Leu-UCRP from cultured endometrial explants collected on days 12–26 of pregnancy (*a*, *b*: $P<0.05$; *b*, *c*: $P<0.01$; *c*, *d*: $P<0.001$). Panel **b** shows the increase in UCRP released into the media following culture of endometrial explants collected on day 12 of the estrous cycle with 25 nM rboIFNT for times specified (*, $P<0.05$). Panels **a** and **b** are from [35]. By using anti-UCRP antibody and anti-ubiquitin antibodies, intracellular UCRP and its conjugates were shown to be up-regulated in response to IFNG and pregnancy [37]. Free ubiquitin and its conjugates did not change in cultured endometrial explants in response to rbIFNT (Panel **c**). This is in contrast to induction of cytosolic UCRP and its conjugates by 12 h followed by release of UCRP into the media by 24 h after culture with rboIFNT (Panels **c** and **d**)

to pregnancy and IFNT in bovine endometrium [5] was found to be the same ubiquitin-like paralog described by the Haas and Knight groups [35] (Fig. 2.1). UCRP increased in endometrium in response to pregnancy from days 14 to 15 and remained detectable through day 26 of pregnancy (Fig. 2.1A). It was inducible in endometrium from non-pregnant cows within 8 h of culture with recombinant bovine IFNT (Fig. 2.1B). Examination of ubiquitin and its conjugates revealed no induction by pregnancy or treatment with IFNT [36, 37] (Fig. 2.1C). Interestingly, ISG15 was not only found as a cytosolic protein in culture endometrial explants, but it was also identified as a secretory product in culture medium (Fig. 2.1D). The presence of secreted ISG15 in uterine flushings from early pregnancy was also

later confirmed, suggesting an extracellular function for ISG15. These data were interpreted to suggest that ISG15 production was a pregnancy- and IFNT-dependent endometrial response.

A bovine endometrial cDNA expression library was constructed and then screened to isolate and sequence ubiquitin and UCRP cDNA [38]. Nucleotide sequence of bovine UCRP was 70 % identical to hUCRP and 30 % identical to a tandem ubiquitin repeat. Use of radio-labeled bUCRP cDNA when screening northern blots revealed that UCRP was detected in bovine endometrium by day 15 and increased to greatest concentrations by days 17–21 of pregnancy [39]. The inferred bUCRP amino acid sequence confirmed retention of the C-terminal Leu-Arg-Gly-Gly amino acids known to be required for conjugation of ubiquitin and ubiquitin paralogues to target proteins [38]. Generation of an anti-bUCRP peptide antibody and use in western blotting of endometrial explants revealed induction of free and conjugated UCRP in response to pregnancy and rbIFNT [37].

Since these early studies, UCRP has been called: ISG15, Ubiquitin-Like Modifier, UCRP1, G1P2, Interferon-Induced 17-kDa/15-kDa Protein, ISG17, Interferon-Stimulated Protein 15 kDa, IFI15, Ubiquitin-Like Protein ISG15, Interferon Alpha-Inducible Protein (Clone IFI-15 K), Interferon-Induced 15 kDa Protein, Ubiquitin Cross-Reactive Protein, and Interferon-Induced 17 kDa Protein. Although the molecular weight more closely approximates 17 kDa across mammalian species, the official protein name is ISG15 and the Entrez Gene name is *ISG15* (Isg15 or *Isg15*; respectively in mice), which will be used from this point forward in this chapter.

Bovine ISG15 and Pregnancy

The b*ISG15* cDNA was used to screen a bovine genomic library and to isolate and sequence the b*ISG15* gene [40]. The b*ISG15* gene, similar to the cDNA, shared about 30 % identity with a tandem ubiquitin repeat and 70 % identity with h*ISG15*. The bISG15 gene promoter has a tandem IFN-stimulated response element (ISRE), which was demonstrated to interact with IFN regulatory factor 1 (IRF1) following culture of bovine endometrial (BEND) cells [41] with rbIFNT. The interaction of this ISRE with BEND cell extracts containing IRF1 corresponded with timing of up-regulation of phosphorylated STAT1 and STAT2 [40].

Because bISG15 was released from cells and also had intracellular ubiquitin-like function when conjugating to cellular proteins, recombinant bISG15 was generated using a *Pichia pastoris* yeast expression system [42] so that it could be more extensively studied. This yeast expression system was used to preclude problems reported when generating ISG15 in bacteria by inherent bacterial carboxypeptidases that cleaved the C-terminus, resulting in removal of active Gly residues. After production and purification of rbISG15 in yeast, C-terminal amino acid sequencing confirmed retention of C-terminal Leu-Arg-Leu-Arg-Gly-Gly residues. Also, the ability of bISG15 to induce up-regulation of IFNG mRNA and protein was demonstrated in cultured peripheral bovine mononuclear cells [42].

A second generation of antibodies against recombinant boISG15 (rboISG15) was developed which included rabbit polyclonal and mouse monocolonal anti-rboISG15 antibodies. Use of a mouse anti-ISG15 monoclonal antibody called 5F10 in immunohistochemistry revealed localization throughout the endometrium from cows on days 18–23 of pregnancy [36]. The most abundant expression of ISG15 localized to the subluminal glandular epithelial cells with lesser, but significant staining in surrounding stromal tissue. There was no staining for ISG15 in endometrium from cows during the estrous cycle and although ubiquitin staining was present in all endometrial sections, ubiquitin was not upregulated in response to pregnancy. A more extensive examination of ISG15 in bovine endometrium on day 18 of pregnancy using transmission electron microscopy and immunogold labeling with anti-bISG15 antibody revealed localization in endometrial glandular epithelium throughout the nucleus, mitochondria, smooth endoplasmic reticulum and plasma membrane [36].

Similar studies in sheep also demonstrated induction of ISG15 in endometrium by pregnancy and IFNT and localization of ISG15 mRNA to endometrial subluminal glandular epithelium and stromal tissue, as well as myometrium [43, 44]. Also, *ISG15* mRNA concentrations have been described to be greater in circulating blood cells during early pregnancy in both sheep and cattle [45, 46]. This prompted studies designed to examine endocrine induction of ISG15 in other ovine tissues during early pregnancy, such as the corpus luteum (CL) [29]. Both ISG15 mRNA and protein were up-regulated in CL in response to early pregnancy. ISG15 localized primarily to the steroidogenic large luteal cells, but also to the gonadotropin-dependent small luteal cells. Recent studies suggest that the inducer of ISG15 in the CL is IFNT that derives from the conceptus, enters uterine vein drainage [28] and then reaches the CL to activate type I IFN receptors, signal transducers such as the STATs and IRFs, and ISG15 gene expression. ISG15 may confer resistance of the CL to PGF through protecting the integrity and steroidogenic machinery and/or attenuating apoptosis in the CL of pregnancy [25, 27].

Structural Features of bISG15

ISG15 interacts with activating enzymes (E1), conjugating enzymes (E2) and ligases (E3) in a manner that is similar, but distinct from that described for ubiquitin [34] (Fig. 2.2A). A ubiquitin activating E1-like (UBE1L) protein was initially described as an enzyme specific for ubiquitin, but later was shown to be specific for ISG15 [47]. Recombinant glutathione S-transferase (GST)-bISG15 was generated using a baculovirus expression system in insect cells, and used to affinity purify interacting proteins from IFNT-treated BEND cells. A 110 kDa protein that bound to GST-ISG15 was purified using this approach [48]. This 110 kDa protein was digested with trypsin and resulting peptides were purified and then submitted to mass spectroscopy determination of mass, which revealed 43–100 % identity to human UBE1L. Bovine UBE1L was localized and upregulated in the endometrium

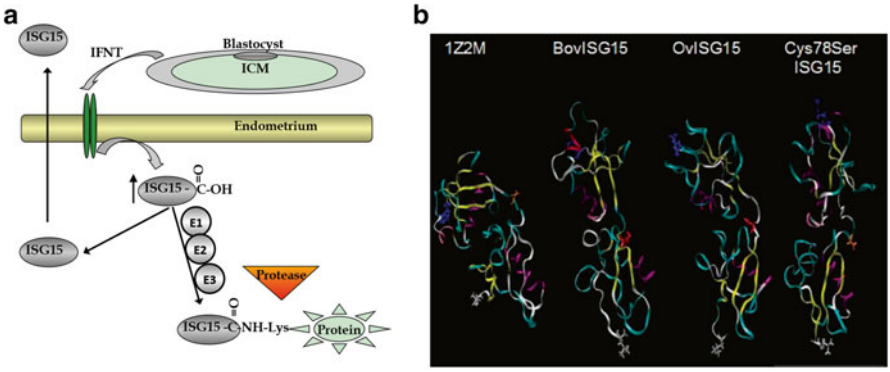


Fig. 2.2 ISG15 conjugation pathway and protein structure. Panel **a** describes release of IFNT from the conceptus, activation of type I IFN receptors on endometrial membranes and synthesis of free ISG15 that can either be released from the cells or can be conjugated to intracellular targeted proteins in an ATP- and multiple enzyme-dependent series of reactions (E1, E2 and E3 activating and conjugating enzymes). After being conjugated to target proteins, ISG15 can be removed through the actions of a de-isgylating enzyme UBP43. Panel **b** shows molecular dynamic simulation of boISG15, ovISG15, and mutant Cys78/Ser ovISG15 based on secondary structure and modeling of human ISG15:1Z2M (from [50]). Note that discrete changes in the hinge region impact structure of both ubiquitin-like domains of ISG15. Bond mode colors represent *blue* for residue 3 in 1Z2M and residue 1 in other ISG15 models, *silver* for residue 154 in 1Z2M protein, 149 in boISG15, 152 in Cys78/Ser ovISG15, *red* for Cys78 and *orange* for Ser 78. *Ribbons* indicate secondary structures as follows: α-helix—*purple*; β-sheet—*yellow*; turn—*cyan*; β-bridge—*tan*; 3–10 α- helix—*mauve*; π-helix coil—*white*

in a manner similar to ISG15 in response to pregnancy and treatment with IFNT. Interestingly, over-expression of GST-ISG15 killed the insect (SF9) cells. Insects do not have an ISG15 ortholog. Thus, the lethal consequences of overexpression of ISG15 might be related to interference by ISG15 with the ubiquitin-dependent pathways that are critical for survival of SF9 cells.

Bovine ISG15 was found to lose stability and three-dimensional structure in solution over time. Intracellular conjugation of ISG15 to target proteins occurs under reducing conditions, and this is consistent with the cytoplasmic environment in general. A critical cysteine residue exists at position 80 in both bovine and ovine ISG15 proteins, and this corresponds to the hinge region between the two ubiquitin-like domains. Cys80 is conserved across mice, humans, bovines and sheep but causes destabilization in hISG15 (actually Cys78 in hISG15) through disulfide bond formation [49]. Modeling of bovine and ovine ISG15 structures was based on human ISG15 (1Z2M) and is shown in Fig. 2.2B. Recombinant rboISG15, roISG15 and corresponding Cys80 → Ser mutants were generated with a C-terminal Arg cap in *Escherichia coli* [50] to protect the C-terminal Gly from endogenous bacterial carboxy-peptidase activity during synthesis [49]. GST-ISG15 was then purified from *E. coli* using affinity chromatography. The C-terminal Arg was removed using carboxy-peptidase B and then correctly processed ISG15 terminating in a C-terminal

Gly was removed from the column using thrombin. Site directed mutagenesis of bISG15 and chemical modification of Cys80 stabilized rboISG15 [50]. The Cys80Ser oISG15 also was more stable and interacted more efficiently with UBE1L. These studies were interpreted to suggest that the hinge region Cys, spacing in the hinge region between the two ubiquitin-like domains and slightly reducing environment are essential to ISG15 structure and are similar to earlier findings by Narasimhan et al. [49]. For example, the hinge region Cys has been shown to form a disulphide bridge with ubiquitin-conjugating enzyme Ubc13 [51]. This non-traditional form of conjugation can be blocked with reducing agents and temporally precedes formation of ISGylated proteins via classical C-terminal Gly-target protein Lys interaction. Other modification to the hinge region Cys may also impact stability and function of ISG15. Post-translational nitrosylation of Cys residues is a recognized cellular response to infection with virus or bacteria. The hinge region Cys of ISG15 may become nitrosylated in response to innate immune responses by nitric oxide, thereby preventing Cys dimerization of ISG15 or interaction of the Cys with other proteins, thus allowing for more free, monomeric ISG15 to interact with targeted proteins via C-terminal ISGylation [52].

Immunoregulatory Action of ISG15

ISG15 is released from human monocytes and lymphocytes in response to culture by Type I IFN [53] and induces the release of IFNG by T lymphocytes, but not natural killer (NK) cells. Bovine ISG15 was first identified in the endometrium as a protein released into the medium following culture of endometrial explants from pregnant cows, as well as from explants or BEND cells from non-pregnant cows treated with bIFNT [5, 35, 41]. For this reason, ISG15 was tested for its ability to induce IFNG in cultured bovine peripheral blood mononuclear cells. While induction of IFNG after culture with ISG15 was detectable, it also was quite variable. Attempts to radioiodinate the single Tyr in bISG15 in radioreceptor assays resulted in very low specific activity which contributed to a lack of sensitivity and problems when trying to demonstrate specific binding to endometrial or PBMC membranes. Likewise, addition of an N-terminal ^{35}S-Met residue also resulted in a protein that had insufficient specific activity for use in receptor binding studies. However, other groups have also suggested an extracellular role of ISG15 in that it: (1) is released from human lymphocytes and monocytes [54] to induce up-regulation of IFNG [53, 55]; (2) induces E-cadherin when cultured with tumor-infiltrating dendritic cells [56]; (3) induces neutrophil-mediated immune responses [57]; and (4) combats mycobacterial infection in humans, presumably through induction of IFNG [58, 59]. This later study implicates an extracellular role for ISG15 in mediating mycobacterial diseases through IFNG, based on a human condition of inherited ISG15 deficiency. A receptor for ISG15 and signal transduction downstream from action of ISG15 on cells has yet to be described. Also, the release of ISG5 from cells is not mediated through a signal peptide; however, non-conventional release might be facilitated through interactions with myxovirus resistance protein MX1 [60].

Mouse ISG15: Studies Relevant to Pregnancy

Because a functional role for ISG15 had not been established in ruminants, our studies of this ubiquitin homolog transitioned to the mouse model, which offered a powerful genetic approach to test gene function. *Isg15* mRNA expression was first examined in the mouse uterus in response to the implanting conceptus [61]. *Isg15* mRNA concentrations increased in the decidualizing stromal compartment (i.e., decidua) between 4.5 and 7.5 days post coitum (dpc) in pregnant mice, and 7.5 and 9.5 dpc mice had significantly greater endometrial *Isg15* mRNA concentrations when compared to pseudopregnant mice (Fig. 2.3A and B). In situ hybridization revealed that *Isg15* mRNA was more extensively localized to antimesometrial decidua on 7.5 dpc and that expression was specific to the pregnancy-induced decidua when compared to the artificially induced deciduoma (Fig. 2.3C) [61, 62]. This confirmed that *Isg15* mRNA was localized to the antimesometrial decidua on 7.5 dpc and is a pregnancy-induced response, rather than a general response to an artificially-induced deciduoma.

Using a cDNA membrane array containing 83 targets, *Isg15* (aka: *G1p2*) was found to be one of five ISGs where steady-state levels in the decidualized uterus depended on the presence of a conceptus [62]. A more extensive screen utilizing the entire mouse genome [63] also identified several up-regulated ISGs in 7.5 dpc decidua when compared to artificially-induced deciduoma. Microarray data were confirmed using RT-PCR for genes known to have biological importance in the endometrium. Semi-quantitative RT-PCR was used to show an increase ($P < 0.05$) in mRNA expression for interferon regulatory factor-8 (*Irf-8*, 1.6-fold), interferon

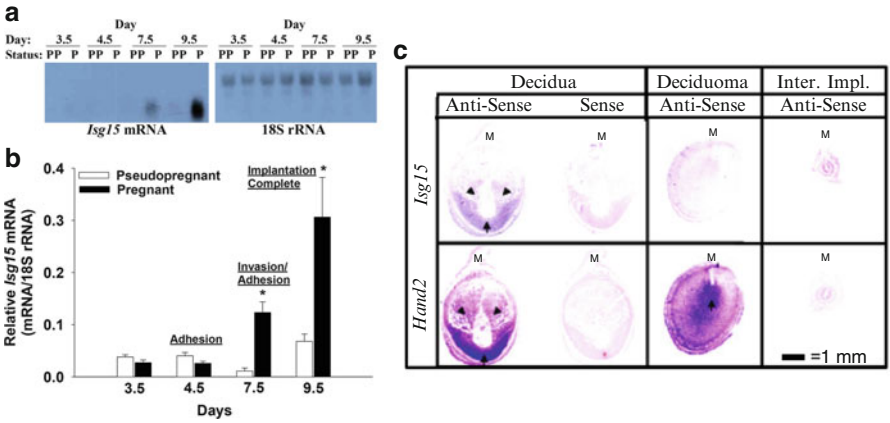


Fig. 2.3 Upregulation of mouse *Isg15* mRNA concentrations in decidua in response to pregnancy. Northern blot revealed induction of ISG15 in mouse uterus in response to pregnancy (P) by day 7.5 post coitum, but not pseudopregnancy (**a** and **b**; *, $P < 0.05$). In situ hybridization of *Isg15* mRNA revealed upregulation in anti-mesometrial decidua in response to pregnancy, but not in response to artificially induced deciduoma (**c**). This is in contrast to up-regulation of *Hand2* mRNA in both decidua and deciduoma. Data are from [61]

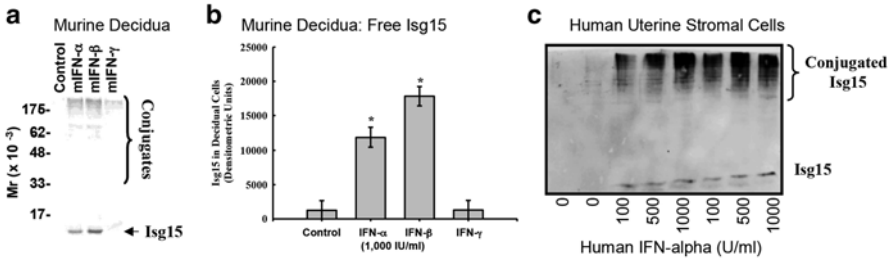


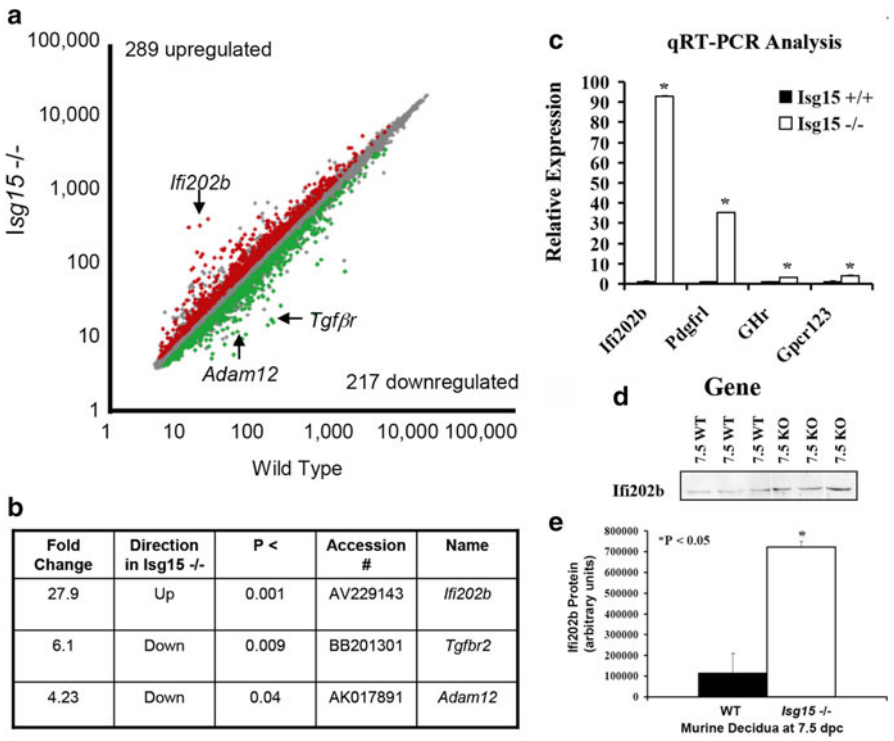
Fig. 2.4 Induction of ISG15 in murine (**a**, **b**) and human (**c**) endometrial stromal cells (Hansen and co-workers, unpublished data). Free ISG15 and its conjugates were up-regulated following 24 h culture of 7.5 dpc murine decidua with IFN-α and IFN-β, but not IFN-γ (Western blot—Panel **a**; Quantitation—Panel **b**; *, P = 0.05). Likewise, human uterine stromal cells responded to culture with IFN-α with a concentration-dependent increase in free ISG15 and ISG15 conjugates

alpha responsive protein (*Iarp*, 2.0-fold), interferon activated gene 202B (*IFI202b*, 1.9-fold), interferon gamma-induced GTPase (*Iigp1*, 1.6-fold), and the interferon stimulated genes *Isg12* (2.5-fold) and *Isg15* (1.6-fold). Uterine ISG15 protein concentrations increased from 4.5 through 7.5 dpc, and then further increased in concentration by 9 dpc. Also culture of murine decidua with type I IFN induced free ISG15 and its conjugated proteins (Fig. 2.4A and B). As was observed in ruminant species, ISGs, including ISG15, are induced in murine endometrium as part of a general interferon/cytokine response to pregnancy.

ISG15 Mouse Mutagenesis Studies

Preliminary study using mutant mice in which the *Isg15* gene was globally deleted (i.e., *Isg15*$^{-/-}$) [64], reported no reproductive phenotype, no impact on antiviral response to vesicular stomatitis virus and lymphocytic chriomenengitis virus, and no apparent impact on IFN signaling through the JAK/STAT signaling pathway. After this primary publication, subsequent studies described *Isg15*$^{-/-}$ mice to be much more susceptible to other viral insults. For example, *Isg15*$^{-/-}$ mice die when challenged with influenza A/WSN/33 and influenza B/Lee/40 [65] viruses. *Isg15*$^{-/-}$ mice also are more susceptible to herpes simplex virus type 1, gammaherpes virus 68 and Sindbis virus infection when compared to WT mice.

Studies in our laboratory using *Isg15*$^{-/-}$ mice resulted in litter sizes at birth that were 50 % smaller when compared to litters from wild type dams (Henkes and Hansen, unpublished results). Despite the fact that surviving *Isg15*$^{-/-}$ pups weighed less at birth, ponderal development following birth was similar when compared to +/+ pups. Loss of embryos in *Isg15*$^{-/-}$mice might be caused by a disruption in gene expression in the maternal decidua. Because *Isg15*$^{-/-}$ embryos died between 7.5 and 12.5 dpc, decidual gene expression was examined on 7.5 dpc. In addition to displaying peak stromal cell decidualization, this stage of early pregnancy was chosen because it represented a time of less trophoblast cell invasion and consequently, less



b

Fold Change	Direction in Isg15 -/-	P <	Accession #	Name
27.9	Up	0.001	AV229143	*Ifi202b*
6.1	Down	0.009	BB201301	*Tgfbr2*
4.23	Down	0.04	AK017891	*Adam12*

Fig. 2.5 Changes in gene expression in decidua from wild type and *Isg15*$^{-/-}$ mice. Scatter plot of 289 up-regulated (*red dots*) and 217 down-regulated (*green dots*) decidual genes that changed 1.5-fold or greater (P<0.05) in *Isg15* null compared to wild type mice. *Arrows* help identifying the *IFI202b*, *Tgfβr* and *Adam12* genes. Panel **b** describes fold-change in *Isg15*$^{-/-}$ mice, P value, accession number and gene name. RT-PCR (Panel **c**) and Western blotting (Panels **d** and **e**) were used to confirm up-regulation of IFI202b in *Isg15*$^{-/-}$ when compared to wild type (WT) 7.5 decidua. Primary pathways impacted because of *Isg15*$^{-/-}$ were signal transduction, immune cell function and vascularization/angiogenesis. Figures adapted from [15]

chance of contamination of uterine decidual tissue mRNA pool with conceptus-derived trophoblast mRNA when compared with later stages of pregnancy. Also, 7.5 dpc was selected because all *Isg15*$^{-/-}$ embryos were found to be alive at this time and it represented a time just prior to loss of pregnancies observed after this time. Following a screen of the Affymetrix GeneChip® Mouse Genome 430 2.0, it was concluded that ISG15 deficiency caused 506 genes to be differentially expressed in decidual tissue, with 289 upregulated (e.g., *Ifi202b*) and 217 down-regulated (e.g., *Adam12* and *Tgfβ2r*) genes [15] (Fig. 2.5A and B).

Ifi202b was identified as the greatest up-regulated gene (28-fold) in the Isg15$^{-/-}$ decidua at 7.5 dpc. The IFI202 family functions in cell-survival and cell cycle regulation. IFI202 is up-regulated by type I IFNs, lipopolysaccharide, and poly-rI:rC treatment [66, 67]. The increase in *Ifi202b* may be a cell-survival mechanism that compensates for some, but not all, of the responses that are mediated through ISG15

for proper decidualization and embryo survival. Down-regulation of decidual *Adam* genes may also be relevant because ADAM8 and ADAM12, localized in mouse implantation sites, are important mediators of uterine remodeling during implantation [68]. Also, low serum ADAM12 concentrations have been associated with pre-eclampsia [69] and intrauterine growth restriction in humans [70]. Whether changes in transcription of these genes are mediated by ISG15 and contribute to establishment of normal litter size and pregnancy remains to be determined.

Ubp43$^{-/-}$ Mouse Studies

ISG15 contains two ubiquitin-like domains connected in tandem. Similar to ubiquitin, ISG15 becomes covalently linked to substrate proteins through cooperative activities of at least three classes of enzymes [71], including an ATP-dependent E1-like enzyme responsible for activation of ISG15 [72], an E2 (UbcH8) conjugating enzyme [73], and an E3 (HERC5 in human and HERC6 in mouse) ligase enzyme [74, 75] (see Fig. 2.2A). ISG15 is removed from targeted proteins through actions of UBP43. A balance of free and conjugated ISG15 is maintained through actions of UBP43, which is up-regulated in response to viral infections, type I IFN and early pregnancy.

Dr. Dong-Er Zhang's laboratory generated and characterized Ubp43$^{-/-}$ mice [76–80]. When *Ubp43* is deleted, dysregulation of the ISG15 system occurs through an abnormal accumulation of conjugated ISG15. The first report using these mice revealed that over-abundance of conjugated ISG15 resulted in live offspring that developed brain cell injury, hypersensitivity to IFN and a 50 % mortality rate by 6 weeks of age [80]. The *Ubp43*$^{-/-}$ brain contained abnormally increased levels of conjugated ISG15, which may have caused ependymal cells to undergo necrosis prior to the development of hydrocephalus. We received these mice from Dr. Zhang and never developed *Ubp43*$^{-/-}$ offspring. By 17.5 dpc, 100 % of null embryos were dead [81]. Examination of *Ubp43*$^{-/-}$ implantation sites on 12.5 dpc revealed disruption of the junctional zone and spongiotrophoblast cells and implantation sites with less vascularization, based on lectin B4 staining, and with greater *Isg15* mRNA and VEGF-A protein concentrations when compared to wild type (WT) placenta. It was concluded that ISG15 and its conjugates were present in implantation sites during mid to late gestation and that deletion of *Ubp43* caused abnormally high concentrations of free and conjugated ISG15 at the feto-maternal interface, which resulted in embryonic death. Because the *Ubp43*$^{-/-}$ mice are from different genetic backgrounds, there may be strain specific genetic modifiers that potentiate lethality of deletion of *Ubp43*. The experiments by Rempel et al. [81], in addition to more recent experiments in Dr. Zhang's laboratory at the Scripps Institute, revealed that under some genetic backgrounds (e.g., backcrossing *Ubp43*$^{+/-}$ into C57 or Balb/c at the F9–F10 generation), *Ubp43*$^{-/-}$ mice were not born. Our studies support the suggestion that dysregulation of ISGylation through abnormal accumulation/over-expression of conjugated ISG15 results in embryo mortality. When combined with the *Isg15*$^{-/-}$ studies, the UBP43 studies also suggest that the ISG15 conjugation

pathway likely plays essential roles on both the maternal and embryonic sides of the implantation interface. Exactly why *Ubp43*$^{-/-}$ results in disrupted implantation sites and embryonic death is unclear, but the consequence of loss of UBP43 is certainly over-accumulation of ISG15 that is conjugated to targeted proteins. This over-accumulation of ISGylated proteins most likely disrupts the balance of free vs. conjugated ISG15, which is maintained by UBP43.

Human and Non-human Primate ISG15 in Decidua in Response to Pregnancy

As with many other mammalian species [4, 35, 36, 39], an early endometrial response to pregnancy in humans and non-human primates [82, 83] is induction of ISG15. ISG15 is induced by IFNs and other cytokines as part of anti-proliferative, antiviral, and inflammatory responses. Localization of ISG15 and ISGylated proteins revealed lack of staining in endometrium from non-pregnant women [82]. However, in response to pregnancy, proteins immune-reactive to anti-ISG15 antibodies were strongly localized to decidual cells. This was apparent in tissues collected from pregnant women on weeks 7–10 and 12 of pregnancy. A similar up-regulation of ISGylated proteins was observed in response to pregnancy in baboon tissues.

Because interleukin 1β (IL1β) induces the decidualization response in primates [84–87], we tested whether ISG15 could be up-regulated in human uterine fibroblast cells (HuF cells) cultured with IL-1β. Treatment of HuF cells with IL-1β resulted in up-regulation of ISG15 and its conjugates. Likewise, culture of HuF cells with type I IFN also resulted in up-regulation of ISG15 and its conjugates (Fig. 2.4C). Other data to support the concept that ISG15 and ISGylation are induced in human decidua in response to pregnancy are provided in a microarray study by Hess et al. where human endometrial stromal cells were decidualized with progesterone and then cultured with media conditioned by human trophoblast [14]. Following microarray analysis, several ISGs, including ISG15 (12-fold up-regulated), were found to be up-regulated following endometrial culture with human trophoblast-conditioned medium. In addition to ISG15, its ISGylation enzymes including initiating enzyme (Ube1l), E2 enzyme (Ubch8) and E3 ligase enzyme (Herc5) were all up-regulated in human uterine stromal cells in response to IL-1β. Exactly why ISG15 is up-regulated in uterine decidua in response to pregnancy and cytokine such as IL and IFN is the focus of future studies.

Summary

Why is ISGylation a conserved response to pregnancy across mammalian species with very divergent forms of embryo implantation and placentation? Many proteins become conjugated with ISG15; yet, a clear role for ISGylation remains to be determined [79, 88–93]. Many of the proposed ISGylated proteins have been tested and

some have been confirmed to be conjugated to ISG15, but the functional roles for ISGylated targets remains to be determined. The amount of targeted protein that becomes ISGylated is low (less that 5 % in some cases), which provides challenges when addressing functional relevance. However, mouse genetic models and human disease conditions with mutations in genes encoding the ISGylation system provide great insights into overall importance of this system to health and well-being. There is no consensus amino acid sequence, to our knowledge, that will allow prediction of proteins that might become ISGylated. Also the massive number of proteins suggested to be conjugated to ISG15 is extensive and these potential targeted proteins have intracellular functions that span the nucleus, cytoplasm, cytoplasmic organelles and plasma membrane.

It is very clear that type I IFNs induce ISGgylation, and this may be important in many species in the endometrium during pregnancy. However, viruses also induce up regulation of ISG15 and ISGylation. If ISGgylation is a general stress response, or subtle innate immune response, perhaps up-regulation in the endometrium helps provide a promp, but not fully activated immune response. This would allow for rapid innate immune response by the mother in the event that infection was approaching the placenta and fetus. Signals from the conceptus, whether IFNT in ruminants or other related cytokines in primates and mice, may coordinate local and systemic immune-modulatory responses to equip the mother when protecting the pregnancy from infection, while also curbing more aggressive adaptive immune responses that could harm the embryo/fetus. This was recently suggested in ruminants [25] and may also have relevance to murine and primate pregnancy.

Acknowledgments This work was supported, in part, by the Traubert Professorship (TH) and grants from the National Institutes of Health, National Institute of Child Health and Development (HD032475 to TH and HD066297 to JP) and Office of the Director (OD010488 to JP), as well as the United States Department of Agriculture National Institute of Food and Agriculture (2011-67015-20067 and 2008-35204-04652) to TH.

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http://www.springer.com/978-1-4939-0816-5

Posttranslational Protein Modifications in the Reproductive System

Sutovsky, P. (Ed.)
2014, XIV, 249 p. 40 illus., 29 illus. in color., Hardcover
ISBN: 978-1-4939-0816-5