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Interplay between Ovine Bone Marrow Stromal Cell Antigen 2/Tetherin and Endogenous Retroviruses[▽]

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Endogenous betaretroviruses (enJSRVs) of sheep are expressed abundantly in the female reproductive tract and play a crucial role in conceptus development and placental morphogenesis. Interestingly, the colonization of the sheep genome by enJSRVs is likely still ongoing. During early pregnancy, enJSRV expression correlates with the production of tau interferon (IFNT), a type I IFN, by the developing conceptus. IFNT is the pregnancy recognition signal in ruminants and possesses potent antiviral activity. In this study, we show that IFNT induces the expression of bone marrow stromal cell antigen 2 (BST2) (also termed CD317/tetherin) both *in vitro* and *in vivo*. The *BST2* gene is duplicated in ruminants. Transfection assays found that ovine BST2 proteins (oBST2A and oBST2B) block release of viral particles produced by intact enJSRV loci and of related exogenous and pathogenic jaagsiekte sheep retrovirus (JSRV). Ovine BST2A appears to restrict enJSRVs more efficiently than oBST2B. *In vivo*, the expression of BST2A/B and enJSRVs in the endometrium increases after day 12 and remains high between days 14 and 20 of pregnancy. *In situ* hybridization analyses found that oBST2A is expressed mainly in the endometrial stromal cells but not in the luminal and glandular epithelial cells, in which enJSRVs are highly expressed. In conclusion, enJSRVs may have coevolved in the presence of oBST2A/B by being expressed in different cellular compartments of the same organ. Viral expression in cells unable to express BST2 may be one of the mechanisms used by retroviruses to escape restriction.

One of the steps of the retrovirus replication cycle is the integration of the reverse-transcribed genome ("provirus") into the host cell genome. During evolution, retroviruses have infected the germ line of their host, leading to stably integrated "endogenous" retroviruses (ERVs) that are transmitted vertically from generation to generation in a Mendelian fashion. Most ERVs have accumulated mutations, substitutions, or deletions that render them defective in establishing a complete viral replication cycle. However, recently integrated ERVs, such as some of the koala and sheep ERVs, possess an intact genomic structure (1, 3, 52). In addition, some of the more ancient ERV loci have maintained genes with open reading frames (ORFs) intact millions of years after their integration in the host genome. This is believed to be the result, in some cases, of positive selection operated by the host because of the beneficial effect afforded by some ERVs. The development of the placenta is one of the best examples offered by the symbiotic relationship between ERVs and their hosts (11, 12).

The sheep genome contains at least 27 copies of endogenous betaretroviruses (enJSRVs) highly related to the exogenous and pathogenic jaagsiekte sheep retrovirus (JSRV) (1, 3, 6, 7, 57). Some of the enJSRV loci are insertionally polymorphic within the sheep population, possess an intact genomic structure with uninterrupted ORFs, and therefore are potentially replication competent (1). The data obtained so far support the idea of an ongoing invasion of the sheep genome by enJSRVs. Some of the biological properties of enJSRVs are vital for their host. Indeed, enJSRV envelope protein (Env) expression is necessary for conceptus (embryo and associated extraembryonic membrane) development and placental morphogenesis and thus mandatory for pregnancy (11, 12). Moreover, some enJSRVs are able to block, at least *in vitro*, several steps of the replication cycle of the exogenous and pathogenic JSRV (1, 2, 29, 30, 47).

Interestingly, enJSRVs are expressed abundantly in the female reproductive tract of sheep, particularly in the endometrium of the uterus (36). The peak of enJSRV expression in the endometrium during early pregnancy coincides with the onset and peak expression of a type I interferon (tau interferon [IFNT]) that functions as the pregnancy recognition signal released from the developing conceptus (43, 46, 48). IFNT has potent antiviral activity both *in vitro* and *in vivo* against a variety of viruses because it induces the same antiviral pathways as other type I IFNs (22, 41, 53). Interestingly, ovine bone marrow stromal cell antigen 2 (BST2)/CD317/tetherin is upregulated by IFNT (8, 17). BST2 has potent antiviral activity (33, 54) and is a type II membrane protein consisting of an N-terminal cytoplasmic tail, a transmembrane domain followed by an extracellular one important for dimerization, and a glycosylphosphatidylinositol (GPI)-linked lipid anchor essential for the viral restriction activity (24, 34). Human BST2 (termed hBST2 in this study and also known as CD317 or

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tetherin) restricts HIV-1 viral particle release in the absence of one of its accessory proteins, Vpu (33, 54). In the presence of BST2 and in the absence of Vpu, nascent HIV-1 virions are retained or "tethered" at the cell surface and subsequently endocytosed (40). However, in the presence of Vpu, restriction by hBST2 is overcome, and HIV-1 particle release is rescued (33, 54). Human BST2 has a broad antiviral spectrum, and several studies found that this protein blocks the viral particle release of many enveloped viruses, including several retroviruses, filoviruses, and arenaviruses (21, 23, 27, 42). In turn, different viruses have evolved a variety of countermeasures to overcome the block induced by BST2 (18, 20, 21, 23, 25, 27, 32, 33, 58).

During early pregnancy, expression of enJSRVs in the endometrium and production of IFNT by the conceptus are correlated. This observation raises the question of how enJSRVs and the IFNT-inducible gene BST2 have coevolved. In this study, we found that IFNT induces expression of oBST2/tetherin both *in vitro* and *in vivo*. The *BST2* gene is duplicated in sheep, and both oBST2A and oBST2B block viral particle release of JSRV and of an intact enJSRV provirus, enJSRV-18. Further, we found that oBST2A is expressed mainly in the stromal cells of the endometrium during early pregnancy but not in the endometrial luminal and glandular epithelial cells that express abundant levels of enJSRVs.

MATERIALS AND METHODS

Plasmids. Plasmids pCMV4JS21 and pCMV5enJS-18 expressing the full-length $JSRV_{21}$ molecular clone and the endogenous provirus enJSRV-18, respectively, have been described (1, 39). The expression plasmids for hBST-2/tetherin tagged with the hemagglutinin (HA) epitope and for an HIV-1 clone deleted of Vpu (HIV-1 ΔVpu) were also described previously (28, 32). Full-length oBST2A cDNA was amplified by reverse transcription (RT)-PCR using total RNA isolated from the endometrium of a day 18 pregnant ewe with a sense primer (5′-GGT CGG CAC CAC TAT TAT GC-3′) and antisense primer (5′-AGT CAG GAA GCC TCA CAC AGG-3′). The ovine *BST2B* gene was first partially amplified from sheep genomic DNA by PCR with a sense primer (5′-GGA GCT TGT CAT TGG TCG GCA CC-3′) and antisense primer (5′-TCA TTT CTT CAG ACA CTT GCA GAT GAT G-3′). Subsequently, in order to confirm its 3′-end sequence, a partial oBST2B cDNA was amplified by RT-PCR from total RNA isolated from sheep choroid plexus cells treated with IFNT. oBST2A and -2B cDNAs were cloned into pCI, with or without an HA epitope tag, resulting in plasmids pCIoBST2A, pCIoBST2B, and pCIoBST2A-HA. The HA tags were inserted into the oBST2A and -2B proteins after amino acid residues 135 and 146, respectively, analogously to the HA-tagged version of hBST2/tetherin, as described previously (28).

Cell cultures, transfections, and viral preparations. Human 293T cells and sheep CPT-Tert cells were cultured in Dulbecco's modified Eagle medium or Iscove's modified Dulbecco's medium, respectively, supplemented with 10% fetal bovine serum at 37°C, 5% CO_2, and 95% humidity. CPT-Tert derives from sheep choroid plexus cells immortalized with the simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (hTERT). For Western blot analysis, viruses were produced by transient transfection of 293T or CPT-Tert cells using the Calphos mammalian transfection kit (Clontech) or Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer's instructions. Viral particle release assays were performed with 293T cells cultured in petri dishes (10-cm diameter) transfected with 2, 4, and 1 μg of the expression plasmids of HIV-1 ΔVpu, JSRV, and enJSRV-18, respectively, either alone or with increasing amounts (0.1 to 1 μg) of the expression plasmids for oBST2A, oBST2B (with or without HA tags), or hBST2 with an HA tag. The amounts of plasmid DNA transfected were the same in each transfection (the empty pCI vector was used to balance each transfection). Viral particle release assays were performed with CPT-Tert cells transfected with 4 μg of the enJSRV-18 expression plasmid and increasing amounts of oBST2A and -2B expression plasmids (0.4 to 4 μg) in order to maintain the ratio of expression plasmids for enJSRV and oBST2 used in the experiments performed with 293T cells. In each transfection, 1 μg pEGFP-C1 (Clontech) was used as a control for transfection efficiency. Cell supernatants were collected at 24 h posttransfection, and viral particles were concentrated by ultracentrifugation. For analysis of intracellular proteins, cells were lysed by standard techniques as described previously (56).

RT-PCR. Expression of *oBST2A* and *oBST2B* in CPT-Tert in the presence of carrier or 10,000 antiviral units (AVU) of IFNT for 24 h was tested by RT-PCR. Total RNA was isolated from CPT-Tert using the Paris kit (Ambion) as recommended by the manufacturers. oBST2A was amplified by RT-PCR using the SuperScript III kit (Invitrogen) and sense primer 5′-ATG ATG GAC AAA TTG GAA GGA TCT-3′ and antisense primer 5′-AAG ACC GCG ATC ACA ACG AAT-3′, while oBST2B was amplified using the sense primer 5′-GTT CCT GAT GCC CAT AGA CGA AGA AGA G-3′ and antisense primer 5′-CGA GTC GTC ACC ACA CCC CCG AAA AAG GA-3′. As an RNA quality control, succinate dehydrogenase (SDHA) cDNA was amplified as described previously (31).

Phylogenetic analysis. Phylogenetic analysis of BST2 proteins from different species was performed using ClustalX, and a phylogenetic tree was built based on the neighbor-joining method, in which the evolutionary rates of the different lineages are free to differ. Clade support was evaluated based on 1,000 bootstrap replicates using the same algorithm.

Western blotting. SDS-PAGE and Western blot analyses were performed with concentrated viral particles and cell lysates (250 μg of protein extracts) as previously described (39, 56). Gag proteins were detected with a rabbit polyclonal serum against the JSRV major capsid protein (CA) or monoclonal antibodies toward HIV-1 p55/p24 (15, 30). The human and ovine BST2 proteins tagged with HA were detected with mouse monoclonal anti-HA antibodies (Covance). The enhanced green fluorescent proteins (eGFP) were revealed using a rabbit polyclonal antibody directed against GFP (ab290; Abcam). Membranes were exposed to the appropriate peroxidase-conjugated secondary antibodies and further developed by chemiluminescence using ECL Plus (Amersham). Western blots were quantified by scanning membranes and measuring chemifluorescence in a Molecular Dynamics Storm 840 imaging system using ImageQuant TL software (Molecular Dynamics). Each experiment (from transfection to Western blotting) was performed independently at least three times.

Animal studies. All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Study 1. At estrus (day 0), ewes were mated to either an intact or vasectomized ram and then hysterectomized ($n = 5$ ewes/day) on days 3, 6, 10, 12, 14, 16, 18, and 20 of the estrous cycle or pregnancy. At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the corpus luteum (CL) were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast Plus (Oxford Labware, St. Louis, MO). Several sections (1 to 1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at −80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80°C for subsequent RNA extraction.

Study 2. Cyclic ewes ($n = 20$) were checked daily for estrus and then ovariectomized and fitted with indwelling uterine catheters on day 5. Ewes were then assigned randomly ($n = 5$ per treatment) to receive daily intramuscular (i.m.) injections of P4 and/or a progesterone receptor and glucorticoid receptor antagonist (mifepristone or RU486; Sigma Chemical Company) and intrauterine (i.u.) infusions of either control (CX) serum proteins and/or recombinant ovine IFNT as follows: (i) 50 mg P4 (days 5 to 16) and 200 μg serum proteins (days 11 to 16) (P4+CX), (ii) P4 and 75 mg RU486 (days 11 to 16) and serum proteins (P4+RU+CX), (iii) P4 and IFNT (2×10^7 antiviral units, days 11 to 16) (P4+IFNT), or (iv) P4 and RU486 and IFNT (P4+RU+IFNT). Steroids were administered i.m. daily in corn oil vehicle. Both uterine horns of each ewe received twice-daily injections of either CX serum proteins (50 μg/horn/injection) or recombinant ovine IFNT (5×10^6 antiviral units/horn/injection). Recombinant ovine IFNT was produced in *Pichia pastoris* and purified as described previously (55). Serum proteins were prepared for intrauterine injection as described previously (44). All ewes were hysterectomized on day 17, and uteri processed as described for study 1.

RNA isolation. Total cellular RNA was isolated from frozen endometrium using Trizol (Gibco-BRL, Bethesda, MD) according to the manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

Slot blot hybridization. Total cellular RNA was isolated from frozen endometrium using Trizol reagent (Gibco-BRL, Bethesda, MD) according to the

manufacturer's instructions. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively. Steady-state levels of *BST2* mRNA were assessed by slot blot hybridization using radiolabeled antisense *oBST2A* and 18S cRNA probes using methods described previously (50). Radiolabeled antisense cRNA probes were generated by *in vitro* transcription using linearized partial plasmid cDNA templates, RNA polymerases, and [α-^{32}P]UTP. Radioactivity associated with slots was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units (RU).

Statistical analyses. Data from slot blot hybridization analyses were subjected to least-squares analysis of variance using the general linear model procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Slot blot hybridization data were corrected for differences in sample loading by using the 18S rRNA data as a covariate. Data were analyzed from study 1 to assess the oBST2A mRNA levels in cyclic and pregnant ewes and from study 2 for the effects of treatment (P4+CX versus P4+IFNT, P4+IFNT versus P4+RU+IFNT, P4+CX versus P4+RU+CX, and P4+RU+CX versus P4+RU+IFNT), as determined using the LSMEANS/PDIFF option of the Statistical Analysis System. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Significance ($P < 0.05$) was determined by probability differences of least-squares means (LSM). Data are presented as LSM with the overall standard error (SE).

qRT-PCR. cDNA was synthesized from endometrial total RNA using random primers (Invitrogen, Carlsbad, CA), oligo(dT) primers, and SuperScript II reverse transcriptase (Invitrogen) as described previously (51). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μl of water, and stored at −20°C for real-time PCR analysis. The PCR analysis of *oBST2A*, *oBST2B*, and *ATP1A1* mRNA was performed using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) with TaqMan universal PCR master mix, No AmpErase UNG (Applied Biosystems), as the detector, according to the manufacturer's recommendations. Briefly, each reaction mixture consisted of 1 μl cDNA template, 0.6 μl of each primer (10 μM), 0.2 μl of the TaqMan probe (10 μM), and 10 μl of the master mix, with double-distilled H_2O (ddH_2O) up to 20 μl. Primer and probe sequences were as follows: oBST2A forward primer, 5′-GCC GAT ATG GAG CGA CTA AG-3′; oBST2A reverse primer, 5′-GCG ATC ACA ACG AAT AGG AA-3′; oBST2A probe, 5′-AGA CAG AAT GAG AAC TC-3′; oBST2B forward primer, 5′-ACC CTG AAG GAC GCT CTG A-3′; oBST2B reverse primer, 5′-GTT CGC CAA CTC TCC CTG A-3′; oBST2B probe, 5′-AAG GAT CAG GCA CGA GTG-3′; ATP1A1 forward primer, 5′-AGA GAG ATA TGC CAA GAT CGT CG-3′; ATP1A1 reverse primer, 5′-GGA GCA CCT TTC ATC ACC AGT A-3′; and ATP1A1 probe, 5′-CTC CAC CAA CAA GTA C-3′. The PCR cycle parameters were 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min for 40 cycles. Template input was optimized from serial dilutions of endometrial cDNA for each gene. The final reactions used 20 ng of cDNA for analysis of *oBST2A*, *oBST2B*, and *ATP1A1*. Data were analyzed using GeneAmp 5700 SDS software (version 2.2; Applied Biosystems). The PCR without template was used as a negative control to verify experimental results. The threshold line was set in the linear region of the plots above the baseline noise, and threshold cycle (C_T) values were determined as the cycle number at which the threshold line crosses the amplification curve.

Quantification of gene amplification was made following RT-PCR by determining the C_T value for reporter fluorescence within the geometric region of the semilog plot generated during the PCR. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. The relative quantification of gene expression across days and treatments was evaluated using the comparative C_T method. The ΔC_T value was determined by subtracting the *ATP1A1* C_T value for each sample from the target C_T value of that sample. Calculation of $\Delta\Delta C_T$ involves using the mean ΔC_T value as an arbitrary calibrator to subtract from all other mean ΔC_T values. Fold changes in the relative mRNA levels for each target gene were then determined by assuming an amplification efficiency of 2 and by applying the equation $2^{-\Delta\Delta CT}$ for each sample.

***In situ* hybridization analysis.** The location of oBST2A or enJSRV *env* mRNAs was determined in sections (5 μm) of sheep uteri by radioactive *in situ* hybridization analysis using methods described previously (50). Archival tissues collected from cyclic and pregnant cow uteri were also used in this analysis. Briefly, deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized oBST2A cDNA or ovine enJSRV *env* partial cDNA (DD54) (50) using *in vitro* transcription with [α-^{35}S]UTP. After being hybridized, washed, and digested with RNase A, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY) and exposed at 4°C for 5 days for oBST2A or 3 days for enJSRV *env*. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under bright-field and dark-field illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

Nucleotide sequence accession number. The cDNAs for oBST2A and -2B have been deposited in GenBank (accession numbers GU376751 and GU376752).

RESULTS

Characterization and cloning of *oBST2A* and *oBST2B*. IFNT is a type I interferon that is secreted by the mononuclear trophoblast cells of the sheep conceptus and functions as the pregnancy recognition signal in this species. Production of IFNT by the conceptus correlates *in vivo* with maximal expression of enJSRVs in the uterine endometria of pregnant ewes (45). Like other type I interferons, IFNT possesses antiviral activity and can upregulate many genes, including viral restriction factors, such as BST2 (8). The bovine genome contains two *BST2*-like genes (referred to here as *bBST2A* and *bBST2B*) located in close proximity on chromosome 7 (13). We performed RT-PCR on RNA isolated from a sheep choroid plexus cell line (CPT-Tert) treated or untreated with IFNT by using oligonucleotide primers designed on the basis of the *bBST2A* and *bBST2B* sequences. Both ovine BST2A and BST2B (oBST2A and -2B) cDNA were detected in IFNT-treated CPT-Tert *in vitro* (Fig. 1A). By sequence analysis, oBST2A and -2B displayed 75.5% identity at the nucleotide level and 48.9% at the amino acid level (Fig. 1B). Phylogenetic analysis of BST2 orthologs showed that oBST2A and bovine BST2A (bBST2A) cluster on a branch different from that for oBST2B and bBST2B (Fig. 1C). These data suggest that duplication of the *BST2* gene occurred before speciation within the *Bovinae* and that the two paralogs then evolved independently.

enJSRVs/JSRV viral particle release is blocked by oBST2A and -2B. As shown in Fig. 1, IFNT induces the expression of oBST2A and -2B in CPT-Tert cells. We next investigated whether oBST2A and -2B blocked release of enJSRVs and the related exogenous JSRV viral particles. We used as a representative example of the enJSRV proviruses enJSRV-18, which has an intact genomic organization and is able to release viral particles *in vitro* (1). In order to check the restriction activity of the two ovine *BST2* genes, we cotransfected both human 293T cells and ovine CPT-Tert with expression plasmids for JSRV or enJSRV-18 and increasing amounts (0.1 to 1 μg) of pCI-oBST2A or pCI-oBST2B (or their respective HA-tagged versions). We equilibrated the amount of expression plasmid DNA used for JSRV and enJSRV-18 in order to express approximately equal levels of Gag (i.e., we used 4 μg of pCMV4JS21 and 1 μg of pCMV5enJS18 in a 10-cm petri dish). Thus, the ratios of the plasmid DNA used to express the retroviruses studied to oBST2A/B were from 40:1 to 4:1 in the experimental JSRV series and varied from 10:1 to 1:1 in the enJSRV-18 series. As shown in Fig. 2, oBST2A was able to block efficiently both JSRV and enJSRV-18 in 293T cells, while oBST2B displayed weaker activity (Fig. 2A to D and F to I). The differences in activity noted between oBST2A and -2B were present with both the untagged and HA-tagged proteins,

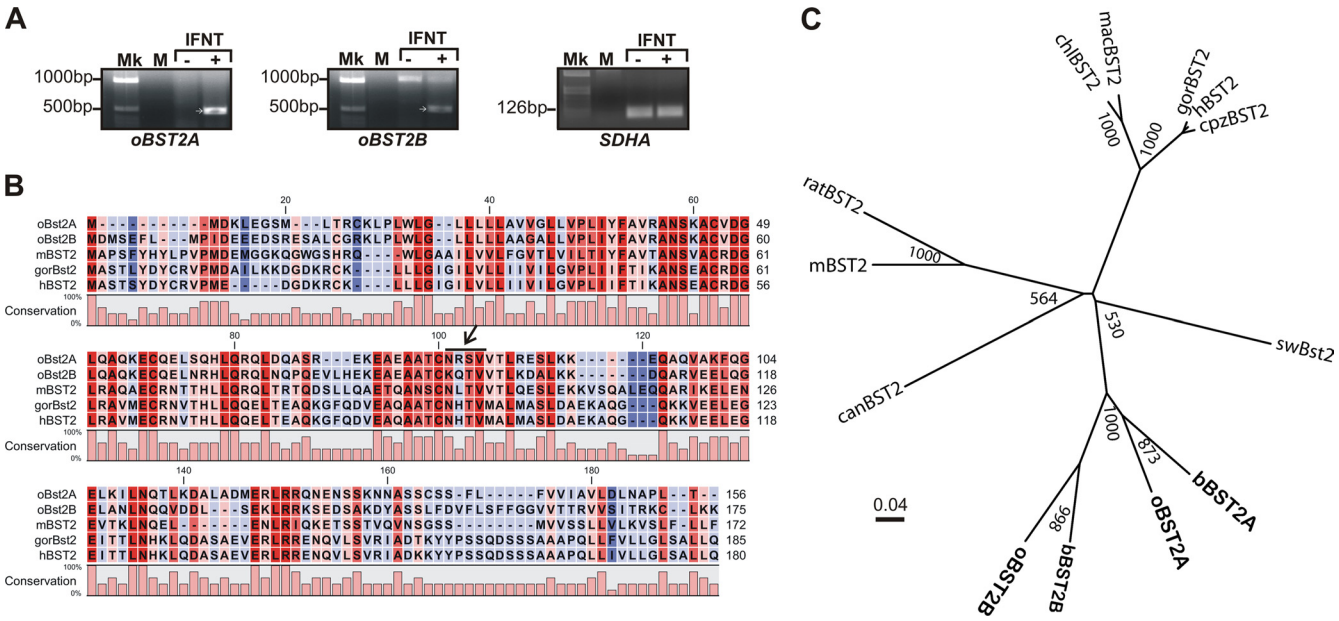

FIG. 1. The *BST2* gene is duplicated in sheep and is induced by IFNT *in vitro*. (A) PCR amplification of two distinct ovine BST2 cDNAs by RT-PCR from total mRNA of sheep choroid plexus (CPT-Tert) cells treated with IFNT. The succinate dehydrogenase (SDHA) cDNA was used as a control of the RNA quality of each sample. Mk indicates the DNA molecular marker, while M is the mock DNA negative control. (B) Alignment of the amino acid sequences of the ovine BST2 proteins (oBST2A and -B), gorilla BST2 (gorBST2), mouse BST2 (mBST2), and human BST2 (hBST2). The arrow indicates a predicted N-glycosylation site that is present in all the above-described sequences, with the exception of oBST2B. (C) Unrooted phylogenetic tree of the BST2 orthologs constructed by using the neighbor-joining method. Clade support was evaluated based on 1,000 bootstrap replicates using the same algorithm. Bootstrap values are indicated. Note that the BST2 paralogs in both bBST2 and oBST2 cluster in different branches of the tree, suggesting that they are conserved and were duplicated before speciation. Each ortholog is differentiated using a letter in front of "BST2" as follows: bBST2, bovine (*Bos taurus*); oBST2, ovine (*Ovis aries*); cpzBST2, chimpanzee (*Pan troglodytes troglodytes*); hBST2, human (*Homo sapiens*); macBST2, macaque (*Macaca mulatta*); chlBST2, vervet monkey (*Chlorocebus aethiops*); gorBST2 (*Gorilla gorilla*); rBST2, rat (*Rattus norvegicus*); mBST2, mouse (*Mus musculus*); canBST2, dog (*Canis familiaris*); swBST2, pig (*Sus scrofa domesticus*).

suggesting that the insertion of the HA epitope did not alter the function of either protein. We also repeated the experiments above with enJSRV-18 in CPT-Tert cells. Interestingly, in these cells both oBST2A and -2B were able to interfere with virus release, although, similarly to what we observed with human cells, the former displayed higher restriction activity than the latter. oBST2A migrates in SDS-PAGE/Western blotting as a triplet of bands, similarly to human BST2 (hBST2), a phenomenon most likely due to N glycosylation of these proteins. Interestingly, oBST2B migrates as a single band and does not possess any potential N-glycosylation site (Fig. 1C).

As expected, both JSRV and enJSRV-18 were blocked by hBST2, and viral particle release was rescued only partially by HIV-1 Vpu (data not shown). The sheep BST2 proteins also possessed broad antiviral activity, as other orthologs of these proteins. oBST2A was able to block in a dose-dependent manner HIV-1 ΔVpu, while oBST2B displayed a less obvious effect. However, the data for the latter are more difficult to interpret, considering that a significant decrease in the levels of HIV-1 Gag in cell lysates was observed with the largest amounts of oBST2B (Fig. 2E and J). Using the same experimental conditions described above, we performed Western blotting assays on lysates and supernatants of cells transfected with expression plasmids for hBST2 and HIV-1 ΔVpu (Fig. 2K). As expected, hBST2 blocked HIV-1 ΔVpu in a dose-dependent manner. We also observed a decrease in the levels of intracellular HIV-1 ΔVpu Gag when cells were cotransfected with the highest concentration of hBST2. This may suggest that within our experimental conditions, higher levels of BST2 might be cytotoxic. However, a fixed amount of an expression plasmid for GFP was also used as an additional control to assess transfection efficiency in all our assays. The levels of GFP, as revealed by Western blotting, were relatively consistent, even in the presence of the highest concentrations of the various BST2 proteins used in our assays. Thus, it is possible that high levels of oBST2B might facilitate intracellular Gag degradation, although this point remains to be experimentally assessed.

oBST2A and -2B are expressed in the endometrium of pregnant ewes. The data presented so far suggested that IFNT can upregulate oBST2A and -2B at least *in vitro*. Here we investigated the effects of IFNT on oBST2A/B *in vivo*. Figure 3 shows a schematic representation of the uterus (Fig. 3A) and of the various early developmental stages of the sheep conceptus. Note that the blastocyte changes morphology by a rapid trophoblast development ("elongation") before attaching to the maternal uterus (Fig. 3B). As mentioned before, IFNT is expressed by the trophoblast of the sheep conceptus (43, 46, 48), and consequently, it is detectable only in pregnant ewes.

We carried out *in situ* hybridization using a *BST2* cRNA antisense probe in order to analyze the ontogeny and location of *BST2* mRNA in the uteri of cyclic and pregnant ewes (Fig. 4). A *BST2* sense cRNA probe was used as a negative control (Fig. 4C and D). *BST2* mRNA was essentially undetectable in

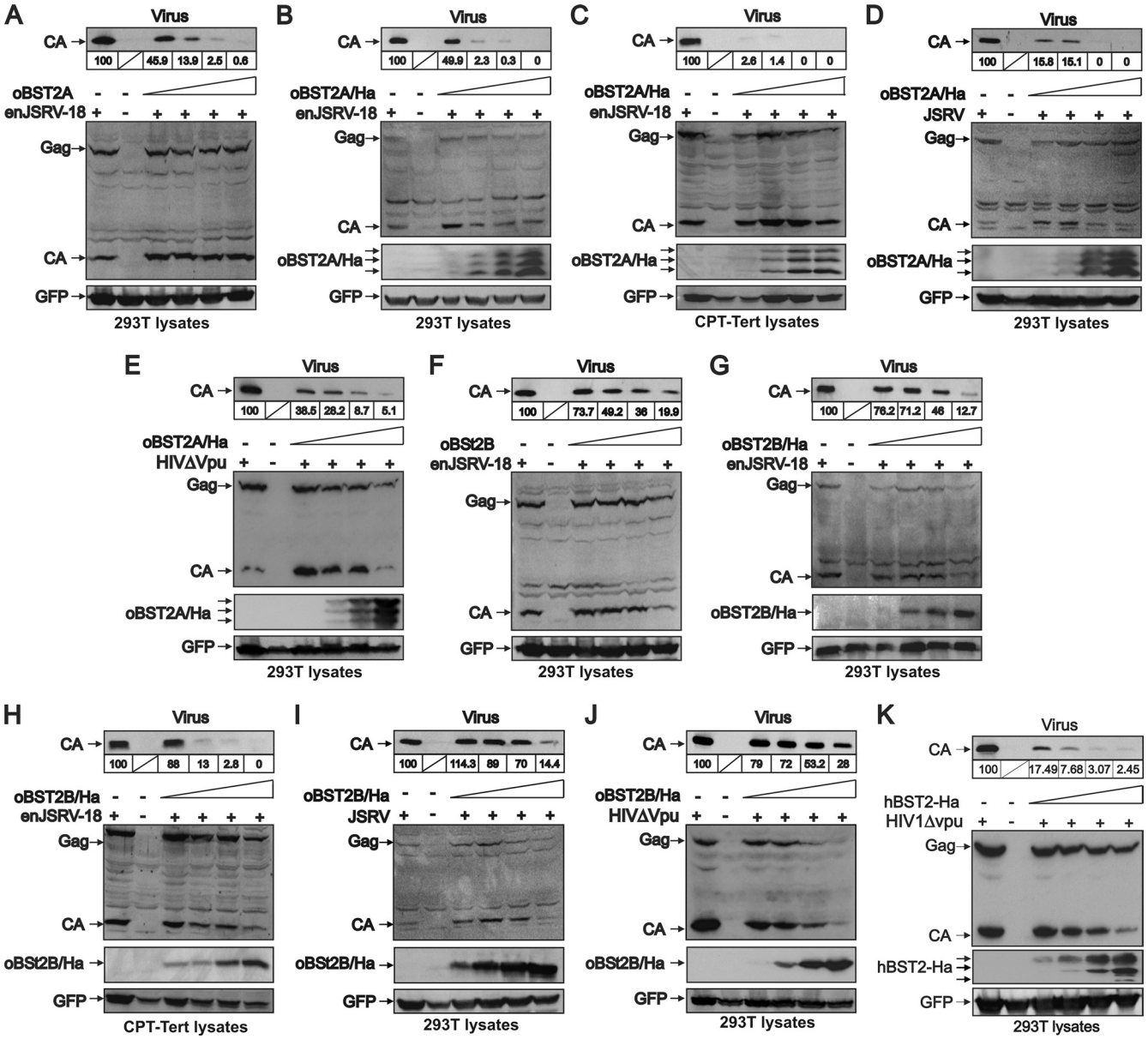

FIG. 2. oBST2A and -2B block viral particle release by JSRV, enJSRV-18, and HIV-1 ΔVpu. Western blotting of concentrated viral particles from supernatants (Virus) and cellular extracts (lysates) of 293T or CPT-Tert cells cotransfected with expression plasmids for viruses and BST2 proteins was as indicated in each panel. Cells were transfected in a 10-cm-diameter petri dish with a fixed amount of expression plasmids for either JSRV (D and I), enJSRV-18 (A to C and F to H), or HIV-1 ΔVpu (E, J, and K), and increasing amounts of expression plasmids for oBST2A, oBST2B (either the untagged or HA-tagged versions), or hBST2 (the HA-tagged version). Blots were incubated with the appropriate antisera, as indicated in each panel. Viral particle release was quantified, and values represent arbitrary units relative to the values of each virus transfected in the absence of BST2 (which was assigned a value of 100). Representative experiments are shown and were repeated at least three times independently.

endometria from cyclic ewes between days 10 and 16 (Fig. 4A, B, E, F, I, and J and data not shown). Similarly, *BST2* mRNA was not detected in the endometria of days 10 and 12 pregnant ewes (Fig. 4G and H and data not shown) but was detected thereafter (days 14 to 20) at increasing levels, specifically in the stroma of the endometrium but not in the luminal epithelia, superficial glandular epithelia, or middle or deep glandular epithelia (Fig. 4L, N, P, R, and T). Moreover, *BST2* mRNA was not detected in the trophoblast of the conceptuses present in uteri from days 18 and 20 of pregnancy (Fig. 4Q and R and data not shown). Note that the probe used for *in situ* hybridization analyses would detect both *oBST2A* and *oBST2B* mRNAs. Essentially, the same results were obtained by *in situ* hybridization in tissue sections obtained from cow uteri. *BST2* expression was detected exclusively in the stroma of the endometrium of cows from day 16 of pregnancy and was not detected in cyclic cows (Fig. 5 and data not shown).

Slot blot hybridization analyses confirmed that steady-state

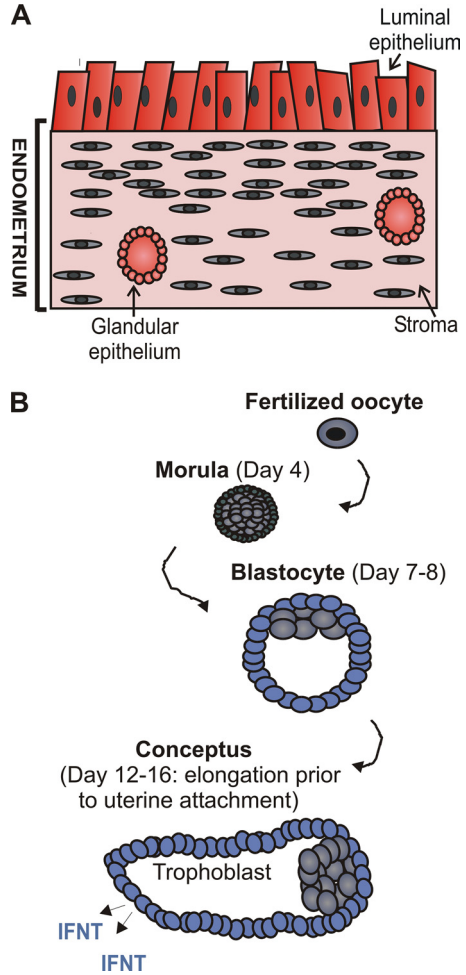

FIG. 3. Schematic diagram of the sheep endometrium and early ovine embryo development. (A) Cross-section of the ovine uterus. The main cellular compartments important for this study are the luminal and glandular epithelia of the endometrium and the stroma. (B) Early embryo development. The fertilized oocyte develops into a multicellular morula at day 4 of pregnancy and then into a hatched spherical blastocyst by days 7 to 8 that consists of an inner cellular mass surrounded by a layer of mononuclear trophoblast cells. Between days 12 and 16, the spherical blastocyst elongates to form a filamentous conceptus that secretes the pregnancy recognition signal, IFNT, and begins the process of implantation.

levels of oBST2A/B mRNA in the endometria of pregnant ewes were considerably higher than those in cyclic (i.e., nonpregnant) ewes (Fig. 6). In cyclic ewes, oBST2A mRNA levels were very low and did not change during the cycle ($P > 0.10$). In contrast, endometrial oBST2A mRNA levels increased substantially in pregnant ewes after day 12, increased ~10-fold on day 14, and remained high to day 20 ($P < 0.01$) (Fig. 6A). In these experiments we used the same probe utilized in the *in situ* hybridization studies described above that do not absolutely discriminate between oBST2A and -2B. By using specific primers for *oBST2A* or *-2B*, we were able to detect a 7-fold increase in the levels of *oBST2A* RNA in the endometrium of day 16 pregnant ewes compared to those in the same tissue collected at day 16 in cyclic ewes (Fig. 6B). A similar pattern was observed for oBST2B, although the increase in the RNA levels in pregnant ewes compared to cyclic ewes was more modest (3.8-fold; Fig. 6B). Thus, the data obtained indicate that oBST2A and -2B are expressed predominantly in the stroma of the endometrium of early pregnant ewes after day 12 of pregnancy. Of note, enJSRV expression is limited to the luminal and glandular epithelia of the ovine uterus and is undetectable in the stroma or myometrium (36, 37, 50) (Fig. 7M to V).

oBST2A expression is induced by IFNT *in vivo*. The temporal increase of oBST2A/B in the endometrium of pregnant sheep beginning on day 12 of pregnancy is correlated directly to the amount of IFNT secreted by the trophectoderm of the rapidly elongating conceptus (Fig. 3B) (49). As reviewed by Spencer et al. (49), many of the genes expressed in the endometrium of early pregnant ewes are regulated by progesterone (P4) produced by the corpus luteum and IFNT from the conceptus trophectoderm. Therefore, we used an *in vivo* hormone replacement model (Fig. 7A) that mimics the effects of P4 and IFNT on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in the ewe (4). Briefly, cyclic ewes were ovariectomized and fitted with intrauterine (i.u.) catheters on day 5, treated with exogenous P4 from days 6 to 17, and given intrauterine infusions of serum proteins as a control (CX) or IFNT from days 11 to 17. The effect of progesterone was assessed by blocking specifically its action through its nuclear receptor using a progesterone receptor antagonist (RU486). As shown in Fig. 7B, treatment of ewes with the RU486 progesterone receptor antagonist did not affect ($P > 0.10$, P4+CX versus P4+RU+CX) endometrial *BST2* mRNA abundance. For ewes receiving P4 alone, intrauterine infusions of recombinant ovine IFNT increased ($P < 0.001$) steady-state levels of *BST2* mRNA in the endometria ($P < 0.001$, P4+CX versus P4+IFNT) (Fig. 7B). Similarly, for ewes receiving P4+RU treatment, intrauterine infusions of recombinant ovine IFNT increased *BST2* mRNA in the endometrium ($P < 0.001$, P4+RU+CX versus P4+RU+IFNT). *In situ* hybridization analyses verified that IFNT increased *BST2* mRNA abundance in the endometrium (Fig. 7C to L). Similarly to that in pregnant ewes, oBST2A mRNA was detected only in the stroma of the endometria from ewes receiving P4 and IFNT (Fig. 7E and F), whereas oBST2A mRNA was detected both in the endometrial stroma and in the luminal epithelium of the uteri of ewes receiving P4, RU486, and IFNT (Fig. 7I and J). Collectively, these results support the idea that IFNT from the elongating conceptus induces expression of oBST2A/B specifically in the stromal cells of the endometrium. In contrast, *in situ* hybridization analyses detected enJSRVs mRNA only in the endometrial luminal and glandular epithelium but not in the stroma or myometrium (Fig. 7M and V). Moreover, the relative abundance of enJSRVs RNA in the endometrial epithelia was unaffected by treatment.

DISCUSSION

In order to further understand the interaction between enJSRVs and their hosts, we investigated the interplay between the ovine *BST2* genes and the endogenous betaretroviruses of sheep (enJSRVs). The sheep genome harbors several copies of enJSRV proviruses that are biologically active, abundantly expressed in the reproductive tract of the ewe, and absolutely

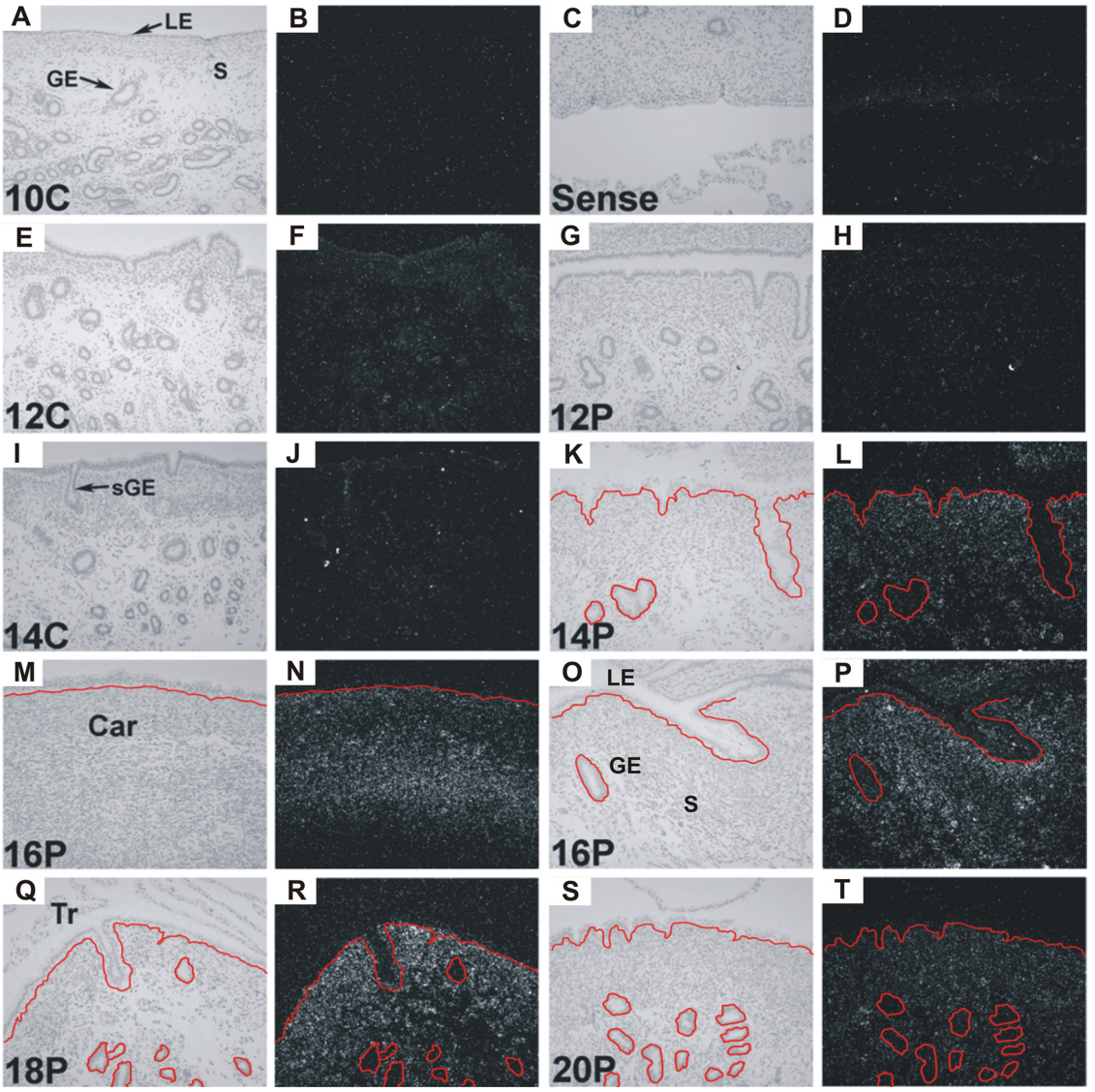

FIG. 4. Expression of oBST2 in the endometrium of cyclic and pregnant ewes. *In situ* hybridization analyses of BST2 mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (10C, 12C, 14C; A, B, E, F, I, and J) and pregnant (12P, 14P, 16P, 18P, and 20P; G, H, K, L, O, P, Q, R, S, and T) ewes were hybridized with radiolabeled antisense ovine *BST2* cRNA probe. Numbers (10C, 12C, 12P, etc.) indicate the day of the cycle (C) or pregnancy (P) of the ewes used in these experiments. (C and D) A sense ovine *BST2* cRNA probe was used as a negative control. Images of representative fields are shown both in bright-field (panels A, C, E, G, I, K, M, O, Q, and S) and dark-field illumination (panels B, D, F, H, J, L, N, P, R, and T). *BST2* mRNA was observed with endometrial stroma of sheep beginning at day 14 of pregnancy (panels L, N, P, R, and T) but not in the luminal epithelia (LE), superficial glandular epithelia (sGE), middle or deep glandular epithelia (GE) of the endometrium, or in the conceptus trophectoderm (Tr). A superimposed red line is shown in panels K to T in order to facilitate the visualization of the LE and GE of the uterus in the sections shown under dark-field illumination. Car, caruncle; S, stroma; Tr, trophectoderm.

required for successful pregnancy in this animal species (3, 11, 12, 14, 35, 38, 57). enJSRVs are highly related to the exogenous and oncogenic JSRV, the causative agent of ovine pulmonary adenocarcinoma, a naturally occurring lung adenocarcinoma of sheep (14, 26, 35). Interestingly, the sheep (and cow) conceptus secretes IFNT, a type I interferon, which functions as a pregnancy recognition signal in this animal species, although it maintains potent antiviral properties. Thus, the sheep represents a unique model system to investigate the coevolution of endogenous and exogenous retroviruses with host antiviral defenses.

The present study found that the *BST2* gene is duplicated in sheep as in cattle. Phylogenetic analysis supports the idea that this duplication occurred approximately 25 million years ago (MYA) before speciation in the *Bovinae* subfamily within the ruminants (19). Thus, duplication of *BST2* predates the initial integration of enJSRVs in the host genome that we estimated occurred between 5 and 7 MYA (1). Both oBST2A and oBST2B blocked viral particle release of enJSRV-18 and the exogenous JSRV. As expected, the antiretroviral activity of these proteins is not specifically targeted to the sheep betaretroviruses, but they have a broader spectrum, considering that

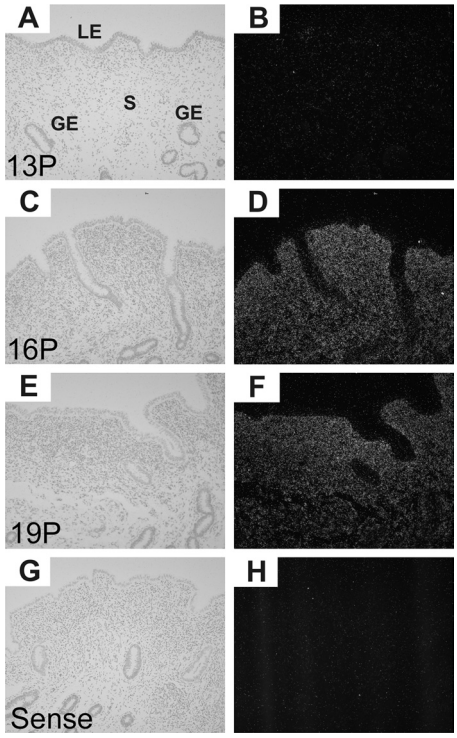

FIG. 5. Expression of oBST2 in the endometrium of pregnant cows. *In situ* hybridization analyses of BST2 mRNA in uteri of pregnant cows. Cross-sections of the uterine wall from pregnant cows (13P, 16P, 19P; A to F) were hybridized with a radiolabeled antisense ovine *BST2* cRNA probe. A sense ovine *BST2* cRNA probe was used as a negative control (G and H). Numbers (13P, 16P, etc.) indicate the day of the pregnancy of the cows used in these experiments. Images of representative fields are shown both in bright-field (A, C, E, and G) and dark-field illumination (B, D, F, and H). *BST2* mRNA was observed in endometrial stroma (S) of cows beginning at day 16 of pregnancy but not in the luminal epithelia (LE) or glandular epithelia (GE) of the endometrium.

HIV-1 ΔVpu can also be blocked by oBST2A/B. These data support the idea that oBST2A has more antiretroviral activity than oBST2B. We speculate that the difference in antiretroviral activities between oBST2A and -2B is due to the proteins *per se* and not to JSRV/enJSRV-18 (viral) factors. Interestingly oBST2B does not possess any predicted N-glycosylation site. We were also unable to identify any GPI anchor signals in the C terminus of this protein. The use of oBST2B mutants and more mechanistic studies will be necessary to determine the molecular basis at the origin of the poor antiviral activity of this protein. Indeed, it is possible that oBST2B may have evolved to serve other functions besides viral restriction.

The two oBST2 proteins displayed stronger restriction activity in sheep cells than in human 293T cells. The difference in these two cell types could be due to ovine-specific cofactors important for oBST2 restriction. As mentioned above, oBST2A inhibits equally well HIV-1 ΔVpu, JSRV, and enJSRV-18 viral particle release. These data support the argument that JSRV/enJSRV-18 do not possess a viral protein counteracting oBST2 restriction.

In the present study, we found that *oBST2A* and *oBST2B* genes were induced by IFNT both *in vitro* and, importantly, *in vivo*. Pregnant sheep showed much higher levels of *oBST2A/B* expression in their endometria than cyclic (i.e., nonpregnant) sheep. The levels of oBST2A/B were correlated to the amount of IFNT produced by the mononuclear trophoblast cells of the elongating conceptus. By using an *in vivo* hormone replacement model (4), we showed that IFNT induces expression of oBST2A/B in the endometrium of the sheep uterus. Indeed, oBST2A is expressed at higher levels than oBST2B. Using a probe that would detect both BST2 mRNAs, *in situ* hybridization analyses revealed that expression of BST2 was restricted to the stromal cells of the endometrium of the ewe. Thus, enJSRVs and BST2 are expressed in different cellular compartments of the same organ. enJSRVs are expressed in the luminal and glandular epithelium of the uterus, while oBST2A and oBST2B are expressed in the stroma. As mentioned above, duplication of the *BST2* gene predates the initial integration of enJSRVs of several million years. Therefore, oBST2A/B could have been one of the driving forces during evolution that shaped the tropism of enJSRV-related exogenous viruses within specific areas of the reproductive tract (i.e., where the BST2 proteins are not expressed). Subsequently, germ line infections by those exogenous viruses led to the selection of enJSRVs present today within the sheep genome. Alternatively, it is possible that sheep have evolved mechanisms to repress BST2 expression in the luminal epithelial cells in order to create a favorable environment for the function of these endogenous retroviruses in host reproductive biology (10–12,

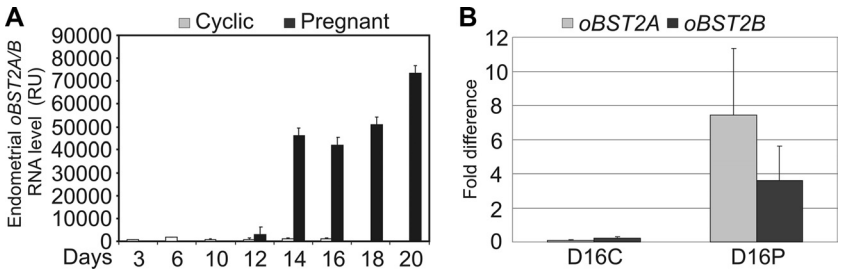

FIG. 6. Relative quantification of BST2A and -2B RNA in cyclic and pregnant ewes. (A) Steady-state levels of BST2A/B mRNAs in endometria from cyclic and early pregnant ewes were determined by slot blot hybridization analysis. In cyclic ewes, *BST2* mRNA was low between days 3 and 16. In contrast, *BST2* mRNA increased ($P < 0.01$) in pregnant ewes between days 12 and 14 and remained maximal between days 14 and 20. Data are expressed as LSM relative units (RU) with standard errors (SE). (B) Relative levels of *BST2A* and *BST2B* mRNA in the endometria of day 16 cyclic and pregnant ewes. The relative mRNA abundance was calculated from the quantitative RT-PCR analysis as described in Materials and Methods.

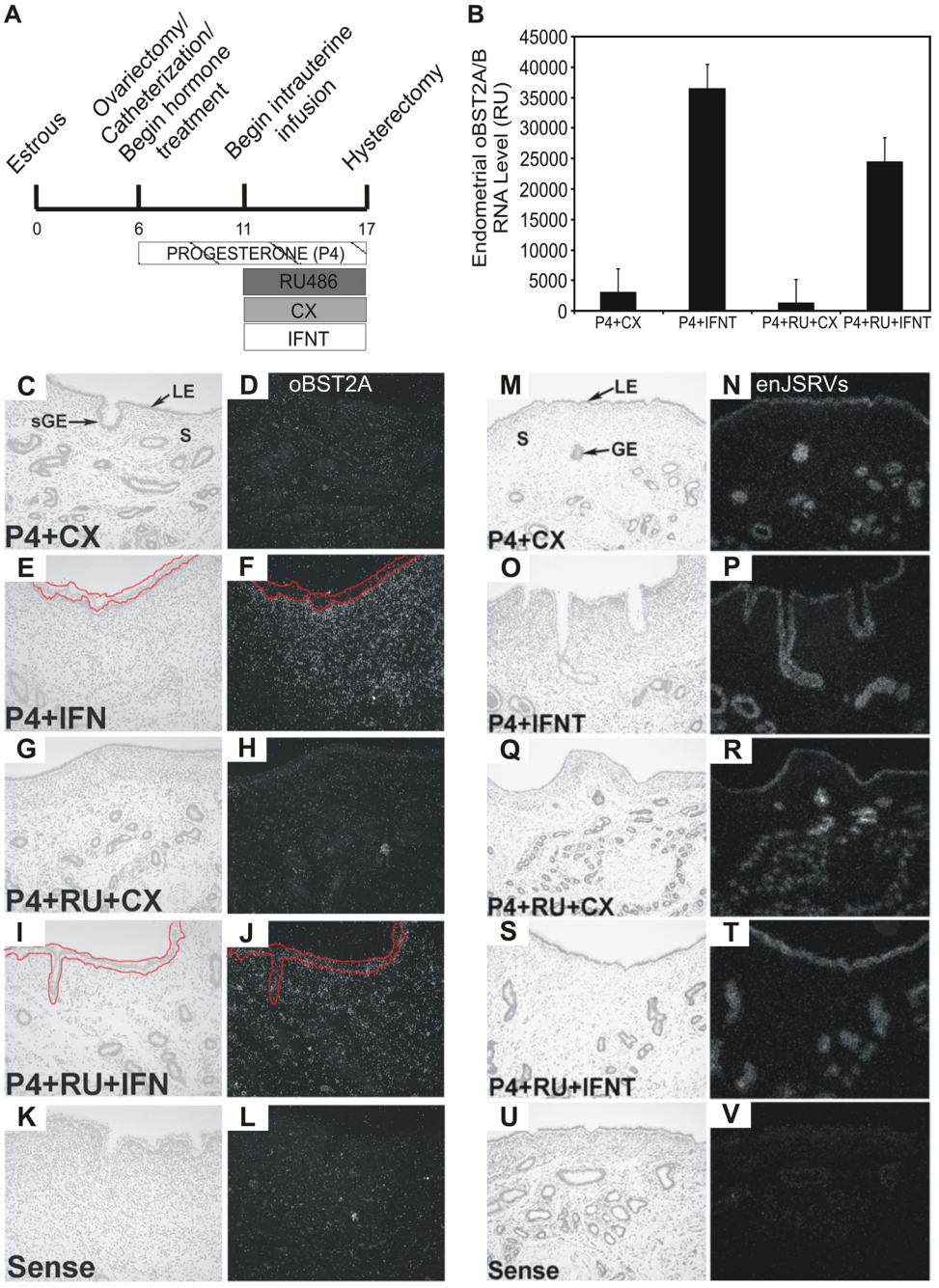

FIG. 7. IFNT induces expression of oBST2 *in vivo*. Effects of progesterone and IFNT on BST2 mRNA in the ovine uterus. (A) Experimental design. See Materials and Methods for complete description. CX, control serum proteins; P4, progesterone; IFNT, recombinant ovine tau interferon; RU, RU486 antiprogestin. (B) Steady-state levels of *oBST2* mRNA in endometria as determined by slot blot hybridization analysis. Intrauterine infusion of IFNT increased *oBST2* mRNA in the endometrium of P4-treated ewes (P4+CX versus P4+IFNT, $P < 0.001$) as well as in ewes receiving the RU486 antiprogestin (P4+IFNT versus P4+RU+IFNT, $P < 0.001$). (C to L) *In situ* hybridization analysis of *oBST2* mRNA expression similar to that described in the legends for Fig. 4 and 5. IFNT increased *BST2* mRNA in a cell-type-specific manner in P4-treated ewes, consistent with increased expression in uteri from day 14, 16, and 18 pregnant ewes. Intrauterine injections of IFNT increased *BST2* mRNA in endometrial stroma but not in LE, GE, or myometrium. A red line has been superimposed to facilitate the visualization of the luminal and glandular epithelia of the uterine stroma. (M to V) *In situ* hybridization analysis of enJSRV *env* RNA expression. The enJSRV *env* RNA was specifically and abundantly present in the endometrial LE and GE but not in the stroma or myometrium.

45, 57). However, we believe that the latter scenario is unlikely because cows, which do not harbor enJSRVs in their genome (1), have a pattern of BST2 expression identical to that of sheep.

In the ovine uterus, the luminal and superficial glandular epithelial cells express a potent transcriptional repressor, IFN regulatory factor 2 (IRF2), that inhibits the activation of many genes induced by IFNT during pregnancy, including many an-

tiviral proteins and classical IFN-stimulated genes (5, 9, 16). Thus, the luminal epithelial cells could have represented a window of opportunity for the replication of enJSRVs. Curiously, we detected oBST2A expression also in the uterine epithelium in sheep treated with progesterone, IFNT, and the progesterone antagonist RU486. It is possible that in the presence of RU486, IRF2 is not expressed or is not active in the luminal epithelium cells of the uterus. Indeed, progesterone normally acts on the stroma cells (which are progesterone receptor positive) and stimulates growth factors, such as fibroblast growth factor 10 (FGF10). In turn, FGF10 acts on the luminal epithelium via its receptor in order to activate signaling pathways that are known to modify transcription factors like IRFs (5, 9). Thus, treatment with RU486 likely deregulates the normal homeostasis of the sheep endometrium and could explain the upregulation of BST2 in the luminal epithelium of ewes infused with IFNT receiving the RU486 antiprogestin.

As mentioned above, the exogenous and pathogenic JSRV is also blocked as efficiently as enJSRV-18 by both oBST2A and -2B. One could have expected that the exogenous JSRV was able to overcome the block induced by BST2. Thus, replication in cellular compartments devoided of BST2 expression may be one of the strategies used by retroviruses to avoid the antiviral response of the host.

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REFERENCES

1. **Arnaud, F., M. Caporale, M. Varela, R. Biek, B. Chessa, A. Alberti, M. Golder, M. Mura, Y. P. Zhang, L. Yu, F. Pereira, J. C. DeMartini, K. Leymaster, T. E. Spencer, and M. Palmarini.** 2007. A paradigm for virus-host coevolution: sequential counter-adaptations between endogenous and exogenous retroviruses. PLoS Pathog. **3:**e170.
2. **Arnaud, F., P. R. Murcia, and M. Palmarini.** 2007. Mechanisms of late restriction induced by an endogenous retrovirus. J. Virol. **81:**11441–11451.
3. **Arnaud, F., M. Varela, T. E. Spencer, and M. Palmarini.** 2008. Coevolution of endogenous betaretroviruses of sheep and their host. Cell. Mol. Life Sci. **65:**3422–3432.
4. **Bazer, F. W., and T. E. Spencer.** 2006. Methods for studying interferon tau stimulated genes. Methods Mol. Med. **122:**367–380.
5. **Bazer, F. W., T. E. Spencer, and T. L. Ott.** 1997. Interferon tau: a novel pregnancy recognition signal. Am. J. Reprod. Immunol. **37:**412–420.
6. **Caporale, M., P. Centorame, A. Giovannini, F. Sacchini, M. Di Ventura, M. De las Heras, and M. Palmarini.** 2005. Infection of lung epithelial cells and induction of pulmonary adenocarcinoma is not the most common outcome of naturally occurring JSRV infection during the commercial lifespan of sheep. Virology **338:**144–153.
7. **Caporale, M., C. Cousens, P. Centorame, C. Pinoni, M. De las Heras, and M. Palmarini.** 2006. Expression of the jaagsiekte sheep retrovirus envelope glycoproteins is sufficient to induce lung tumor in sheep. J. Virol. **80:**8030–8037.
8. **Chen, Y., E. Antoniou, Z. Liu, L. B. Hearne, and R. M. Roberts.** 2007. A microarray analysis for genes regulated by interferon-tau in ovine luminal epithelial cells. Reproduction **134:**123–135.
9. **Choi, Y., G. A. Johnson, R. C. Burghardt, L. R. Berghman, M. M. Joyce, K. M. Taylor, M. D. Stewart, F. W. Bazer, and T. E. Spencer.** 2001. Interferon regulatory factor-two restricts expression of interferon-stimulated genes to the endometrial stroma and glandular epithelium of the ovine uterus. Biol. Reprod. **65:**1038–1049.
10. **Dunlap, K. A., M. Palmarini, D. L. Adelson, and T. E. Spencer.** 2005. Sheep endogenous betaretroviruses (enJSRVs) and the hyaluronidase 2 (HYAL2) receptor in the ovine uterus and conceptus. Biol. Reprod. **73:**271–279.
11. **Dunlap, K. A., M. Palmarini, and T. E. Spencer.** 2006. Ovine endogenous betaretroviruses (enJSRVs) and placental morphogenesis. Placenta **27**(Suppl. A)**:**S135–S140.
12. **Dunlap, K. A., M. Palmarini, M. Varela, R. C. Burghardt, K. Hayashy, J. L. Farmer, and T. E. Spencer.** 2006. Endogenous retroviruses regulate periimplantation conceptus growth and differentiation. Proc. Natl. Acad. Sci. U. S. A. **103:**14390–14395.
13. **Elsik, C. G., R. L. Tellam, K. C. Worley, R. A. Gibbs, D. M. Muzny, G. M. Weinstock, D. L. Adelson, E. E. Eichler, L. Elnitski, R. Guigo, D. L. Hamernik, S. M. Kappes, H. A. Lewin, D. J. Lynn, F. W. Nicholas, A. Reymond, M. Rijnkels, L. C. Skow, E. M. Zdobnov, L. Schook, J. Womack, T. Alioto, S. E. Antonarakis, A. Astashyn, C. E. Chapple, H. C. Chen, J. Chrast, F. Camara, O. Ermolaeva, C. N. Henrichsen, W. Hlavina, Y. Kapustin, B. Kiryutin, P. Kitts, F. Kokocinski, M. Landrum, D. Maglott, K. Pruitt, V. Sapojnikov, S. M. Searle, V. Solovyev, A. Souvorov, C. Ucla, C. Wyss, J. M. Anzola, D. Gerlach, E. Elhaik, D. Graur, J. T. Reese, R. C. Edgar, J. C. McEwan, G. M. Payne, J. M. Raison, T. Junier, E. V. Kriventseva, E. Eyras, M. Plass, R. Donthu, D. M. Larkin, J. Reecy, M. Q. Yang, L. Chen, Z. Cheng, C. G. Chitko-McKown, G. E. Liu, L. K. Matukumalli, J. Song, B. Zhu, D. G. Bradley, F. S. Brinkman, L. P. Lau, M. D. Whiteside, A. Walker, T. T. Wheeler, T. Casey, J. B. German, D. G. Lemay, N. J. Maqbool, A. J. Molenaar, S. Seo, P. Stothard, C. L. Baldwin, R. Baxter, C. L. Brinkmeyer-Langford, W. C. Brown, C. P. Childers, T. Connelley, S. A. Ellis, K. Fritz, E. J. Glass, C. T. Herzig, A. Iivanainen, K. K. Lahmers, A. K. Bennett, C. M. Dickens, J. G. Gilbert, D. E. Hagen, H. Salih, J. Aerts, A. R. Caetano, et al.** 2009. The genome sequence of taurine cattle: a window to ruminant biology and evolution. Science **324:**522–528.
14. **Fan, H.** 2003. Jaagsiekte sheep retrovirus and lung cancer. Springer-Verlag, Berlin, Germany.
15. **Ferns, R. B., R. S. Tedder, and R. A. Weiss.** 1987. Characterization of monoclonal antibodies against the human immunodeficiency virus (HIV) gag products and their use in monitoring HIV isolate variation. J. Gen. Virol. **68**(6)**:**1543–1551.
16. **Fleming, J. A., G. Song, Y. Choi, T. E. Spencer, and F. W. Bazer.** 2009. Interferon regulatory factor 6 (IRF6) is expressed in the ovine uterus and functions as a transcriptional activator. Mol. Cell. Endocrinol. **299:**252–260.
17. **Gray, C. A., C. A. Abbey, P. D. Beremand, Y. Choi, J. L. Farmer, D. L. Adelson, T. L. Thomas, F. W. Bazer, and T. E. Spencer.** 2006. Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. Biol. Reprod. **74:**383–394.
18. **Gupta, R. K., P. Mlcochova, A. Pelchen-Matthews, S. J. Petit, G. Mattiuzzo, D. Pillay, Y. Takeuchi, M. Marsh, and G. J. Towers.** 28 October 2009. Simian immunodeficiency virus envelope glycoprotein counteracts tetherin/BST-2/CD317 by intracellular sequestration. Proc. Natl. Acad. Sci. U. S. A. [Epub ahead of print.]
19. **Hernandez Fernandez, M., and E. S. Vrba.** 2005. A complete estimate of the phylogenetic relationships in Ruminantia: a dated species-level supertree of the extant ruminants. Biol. Rev. Camb. Philos. Soc. **80:**269–302.
20. **Jia, B., R. Serra-Moreno, W. Neidermyer, A. Rahmberg, J. Mackey, I. B. Fofana, W. E. Johnson, S. Westmoreland, and D. T. Evans.** 2009. Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST2. PLoS Pathog. **5:**e1000429.
21. **Jouvenet, N., S. J. Neil, M. Zhadina, T. Zang, Z. Kratovac, Y. Lee, M. McNatt, T. Hatziioannou, and P. D. Bieniasz.** 2009. Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin. J. Virol. **83:**1837–1844.
22. **Juste, R. A., T. L. Ott, J. Kwang, F. W. Bazer, and A. de La Concha-Bermejillo.** 2000. Effects of recombinant ovine interferon-tau on ovine lentivirus replication and progression of disease. J. Gen. Virol. **81:**525–532.
23. **Kaletsky, R. L., J. R. Francica, C. Agrawal-Gamse, and P. Bates.** 2009. Tetherin-mediated restriction of filovirus budding is antagonized by the Ebola glycoprotein. Proc. Natl. Acad. Sci. U. S. A. **106:**2886–2891.
24. **Kupzig, S., V. Korolchuk, R. Rollason, A. Sugden, A. Wilde, and G. Banting.** 2003. Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology. Traffic **4:**694–709.
25. **Le Tortorec, A., and S. J. Neil.** 2009. Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. J. Virol. **83:**11966–11978.
26. **Liu, S. L., and A. D. Miller.** 2007. Oncogenic transformation by the jaagsiekte sheep retrovirus envelope protein. Oncogene **26:**789–801.
27. **Mansouri, M., K. Viswanathan, J. L. Douglas, J. Hines, J. Gustin, A. V. Moses, and K. Fruh.** 2009. Molecular mechanism of BST2/tetherin downregulation by K5/MIR2 of Kaposi's sarcoma-associated herpesvirus. J. Virol. **83:**9672–9681.
28. **McNatt, M. W., T. Zang, T. Hatziioannou, M. Bartlett, I. B. Fofana, W. E. Johnson, S. J. Neil, and P. D. Bieniasz.** 2009. Species-specific activity of HIV-1 Vpu and positive selection of tetherin transmembrane domain variants. PLoS Pathog. **5:**e1000300.
29. **Mura, M., P. Murcia, M. Caporale, T. E. Spencer, K. Nagashima, A. Rein, and M. Palmarini.** 2004. Late viral interference induced by transdominant Gag of an endogenous retrovirus. Proc. Natl. Acad. Sci. U. S. A. **101:**11117–11122.

30. **Murcia, P. R., F. Arnaud, and M. Palmarini.** 2007. The transdominant endogenous retrovirus enJS56A1 associates with and blocks intracellular trafficking of the JSRV Gag. J. Virol. **81:**1762–1772.
31. **Nalubamba, K. S., A. G. Gossner, R. G. Dalziel, and J. Hopkins.** 2007. Differential expression of pattern recognition receptors in sheep tissues and leukocyte subsets. Vet. Immunol. Immunopathol. **118:**252–262.
32. **Neil, S. J., S. W. Eastman, N. Jouvenet, and P. D. Bieniasz.** 2006. HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. PLoS Pathog. **2:**e39.
33. **Neil, S. J., T. Zang, and P. D. Bieniasz.** 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature **451:**425–430.
34. **Ohtomo, T., Y. Sugamata, Y. Ozaki, K. Ono, Y. Yoshimura, S. Kawai, Y. Koishihara, S. Ozaki, M. Kosaka, T. Hirano, and M. Tsuchiya.** 1999. Molecular cloning and characterization of a surface antigen preferentially overexpressed on multiple myeloma cells. Biochem. Biophys. Res. Commun. **258:**583–591.
35. **Palmarini, M., and H. Fan.** 2003. Molecular biology of jaagsiekte sheep retrovirus. Curr. Top. Microbiol. Immunol. **275:**81–115.
36. **Palmarini, M., C. A. Gray, K. Carpenter, H. Fan, F. W. Bazer, and T. E. Spencer.** 2001. Expression of endogenous betaretroviruses in the ovine uterus: effects of neonatal age, estrous cycle, pregnancy, and progesterone. J. Virol. **75:**11319–11327.
37. **Palmarini, M., C. Hallwirth, D. York, C. Murgia, T. de Oliveira, T. Spencer, and H. Fan.** 2000. Molecular cloning and functional analysis of three type D endogenous retroviruses of sheep reveal a different cell tropism from that of the highly related exogenous jaagsiekte sheep retrovirus. J. Virol. **74:**8065–8076.
38. **Palmarini, M., M. Mura, and T. E. Spencer.** 2004. Endogenous betaretroviruses of sheep: teaching new lessons in retroviral interference and adaptation. J. Gen. Virol. **85:**1–13.
39. **Palmarini, M., J. M. Sharp, M. De las Heras, and H. Fan.** 1999. Jaagsiekte sheep retrovirus is necessary and sufficient to induce a contagious lung cancer in sheep. J. Virol. **73:**6964–6972.
40. **Perez-Caballero, D., T. Zang, A. Ebrahimi, M. W. McNatt, D. A. Gregory, M. C. Johnson, and P. D. Bieniasz.** 2009. Tetherin inhibits HIV-1 release by directly tethering virions to cells. Cell **139:**499–511.
41. **Rogez, C., M. Martin, N. Dereuddre-Bosquet, J. Martal, D. Dormont, and P. Clayette.** 2003. Anti-human immunodeficiency virus activity of tau interferon in human macrophages: involvement of cellular factors and beta-chemokines. J. Virol. **77:**12914–12920.
42. **Sakuma, T., T. Noda, S. Urata, Y. Kawaoka, and J. Yasuda.** 2009. Inhibition of Lassa and Marburg virus production by tetherin. J. Virol. **83:**2382–2385.
43. **Spencer, T. E., and F. W. Bazer.** 1996. Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. Endocrinology **137:**1144–1147.
44. **Spencer, T. E., N. H. Ing, T. L. Ott, J. S. Mayes, W. C. Becker, G. H. Watson, M. A. Mirando, and F. W. Brazer.** 1995. Intrauterine injection of ovine interferon-tau alters oestrogen receptor and oxytocin receptor expression in the endometrium of cyclic ewes. J. Mol. Endocrinol. **15:**203–220.
45. **Spencer, T. E., G. A. Johnson, F. W. Bazer, R. C. Burghardt, and M. Palmarini.** 2007. Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reprod. Fertil. Dev. **19:**65–78.
46. **Spencer, T. E., M. A. Mirando, J. S. Mayes, G. H. Watson, T. L. Ott, and F. W. Bazer.** 1996. Effects of interferon-tau and progesterone on oestrogen-stimulated expression of receptors for oestrogen, progesterone and oxytocin in the endometrium of ovariectomized ewes. Reprod. Fertil. Dev. **8:**843–853.
47. **Spencer, T. E., M. Mura, C. A. Gray, P. J. Griebel, and M. Palmarini.** 2003. Receptor usage and fetal expression of ovine endogenous betaretroviruses: implications for coevolution of endogenous and exogenous retroviruses. J. Virol. **77:**749–753.
48. **Spencer, T. E., T. L. Ott, and F. W. Bazer.** 1996. tau-Interferon: pregnancy recognition signal in ruminants. Proc. Soc. Exp. Biol. Med. **213:**215–229.
49. **Spencer, T. E., O. Sandra, and E. Wolf.** 2008. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction **135:**165–179.
50. **Spencer, T. E., A. G. Stagg, M. M. Joyce, G. Jenster, C. G. Wood, F. W. Bazer, A. A. Wiley, and F. F. Bartol.** 1999. Discovery and characterization of endometrial epithelial messenger ribonucleic acids using the ovine uterine gland knockout model. Endocrinology **140:**4070–4080.
51. **Stewart, M. D., G. A. Johnson, C. A. Gray, R. C. Burghardt, L. A. Schuler, M. M. Joyce, F. W. Bazer, and T. E. Spencer.** 2000. Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. Biol. Reprod. **62:**1779–1789.
52. **Tarlinton, R. E., J. Meers, and P. R. Young.** 2006. Retroviral invasion of the koala genome. Nature **442:**79–81.
53. **Tennakoon, D. K., R. Smith, M. D. Stewart, T. E. Spencer, M. Nayak, and C. J. Welsh.** 2001. Ovine IFN-tau modulates the expression of MHC antigens on murine cerebrovascular endothelial cells and inhibits replication of Theiler's virus. J. Interferon Cytokine Res. **21:**785–792.
54. **Van Damme, N., D. Goff, C. Katsura, R. L. Jorgenson, R. Mitchell, M. C. Johnson, E. B. Stephens, and J. Guatelli.** 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. Cell Host Microbe **3:**245–252.
55. **Van Heeke, G., T. L. Ott, A. Strauss, D. Ammaturo, and F. W. Bazer.** 1996. High yield expression and secretion of the ovine pregnancy recognition hormone interferon-tau by Pichia pastoris. J. Interferon Cytokine Res. **16:**119–126.
56. **Varela, M., Y. H. Chow, C. Sturkie, P. Murcia, and M. Palmarini.** 2006. Association of RON tyrosine kinase with the jaagsiekte sheep retrovirus envelope glycoprotein. Virology **350:**347–357.
57. **Varela, M., T. E. Spencer, M. Palmarini, and F. Arnaud.** 2009. Friendly viruses: the special relationship between sheep and retroviruses. Ann. N. Y. Acad. Sci. **1178:**157–172.
58. **Zhang, F., S. J. Wilson, W. C. Landford, B. Virgen, D. Gregory, M. C. Johnson, J. Munch, F. Kirchhoff, P. D. Bieniasz, and T. Hatziioannou.** 2009. Nef proteins from simian immunodeficiency viruses are tetherin antagonists. Cell Host Microbe **6:**54–67.