PORTLAND PRESS

Research Article

Novel endogenous retrovirus-derived transcript expressed in the bovine placenta is regulated by WNT signaling

Toshihiro Sakurai[1], So Nakagawa[2], Hanako Bai[3], Rulan Bai[4], Kazuya Kusama[4], Atsushi Ideta[5], Yoshito Aoyagi[5], Kazuyuki Kaneko[6], Kosuke Iga[7], Jiro Yasuda[8], Takayuki Miyazawa[9] and Kazuhiko Imakawa[4]

[1]Department of Occupational and Environment Health, Tokyo University of Science, Chiba 278-0022, Japan; [2]Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa 259-1193, Japan; [3]Department of Animal Science, Hokkaido University, Hokkaido 060-8589, Japan; [4]Animal Resource Science Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Ibaraki 319-0206, Japan; [5]Zen-noh Embryo Transfer Center, Kamishihoro, Hokkaido 080-1407, Japan; [6]Laboratory of Theriogenology, Department of Veterinary Medicine, Azabu University, Kanagawa 252-5201, Japan; [7]Tohoku Agricultural Research Center, National Agriculture and Food Research Organization, Iwate 020-0198, Japan; [8]Department of Emerging Infectious Diseases, Institute for Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan; [9]Laboratory of Signal Transduction, Department of Cell Biology, Institute for Viral Research, Kyoto University, Kyoto 606-8507, Japan

Correspondence: Kazuhiko Imakawa (akaz@mail.ecc.u-tokyo.ac.jp)

OPEN ACCESS

Endogenous retroviruses (ERVs) are involved in placentation; perhaps, the most well-known *ERV*s are the syncytins, actively transcribed *env* genes involved in cell–cell fusion and possible morphological variations. However, ERVs other than syncytins that play an important role in placental development have not been well characterized. To identify *ERV* genes expressed during the onset of placentation in the bovine species, we characterized the expression profiles of bovine conceptus transcripts during the peri-attachment period using RNA-seq analysis, and confirming some candidates through real-time PCR. Using *in silico* and PCR analyses, we identified a novel *ERV* proviral sequence derived from a *gag* region, designated bovine endogenous retroviruses (BERV)-K3, containing *Gag*_p10 and *Gag*_p24, zinc finger domain. Initial expression of this *ERV* in bovine conceptuses was on day 20 (day 0 = day of estrus), soon after conceptus attachment to the endometrial epithelium, and its high placental expression was maintained up to the middle of pregnancy. The *BERV-K3* transcript was also found in the uterine luminal and glandular epithelia, liver, kidney, intestine, and skin. *BERV-K3* is located on chromosome 7 and integrated within *LOC100848658*, from which noncoding RNA could be transcribed. Furthermore, the expression of endogenous *BERV-K3* in bovine trophoblast cell lines was induced by a WNT agonist, a signaling system common to genes expressed in placentas. These data support the argument that during the evolutionary process, mammals incorporated not only similar *ERV* sequences, but also *ERV*s unique to individual species. BERV-K3 is in the latter case, likely providing functions unique to ruminant gestation.

Introduction

The endogenous retroviruses (ERVs) are thought to be derived from ancient viral infections of germ cells or their precursors with a retrovirus in mammals and other vertebrates [1]. Indeed, sequences of retroviral origins make up 8 % of the human genome, 10% of the mouse genome, and 18% of the bovine genome [2–4]. Most *ERV*s have been inactivated by insertions, deletions, and substitutions of nucleotides, and/or epigenetic modifications such as DNA methylation and histone modifications. However, a few open-reading frames (ORFs) of *ERV* sequences remain intact and are expressed as virus-derived proteins in the host cells [5].

The placenta is remarkably distinct among mammalian species, suggesting a history of rapid evolutionary diversification, resulting from the genes acquired in individual species. It has become apparent that *ERV* genes play an important role in the development of the placenta and the trophoblast cell lineage in mammalian species, and that during evolution different species may have utilized ERVs of the same as well as different origins. Indeed, different *env* genes, syncytins, essential for placental morphogenesis have been independently integrated into the genome of humans [6–10], mice [11], rabbits [12], dogs [13], cats [13], sheep [14–18], and cattle [19–23], sheep [21–23], the Rodentia squirrel-related clade [24], Afrotherian tenrecs [25], and marsupials [26]. All identified syncytin *env* genes exist in different genome sequences and chromosomal locations among species, but the functions such as cell fusion and immune suppression are all shared in mammals. However, the exact evolutionary pathways and the extent to which ERVs function in placental development are still unclear.

It has been determined that the WNT signaling pathway is an important regulator of embryo/conceptus and maternal interaction such as implantation and placental development in mice, sheep, cow, and humans [27–30]. The WNT can induce two downstream signaling cascades, known as the canonical and noncanonical pathways [31]. The canonical WNT pathway is activated when WNT binds to Frizzled (*FZD*) family receptors and low-density lipoprotein receptor-related protein (*LRP*) 5/6 coreceptors. This, in turn, leads to cellular accumulation and nuclear translocation of β-catenin (*CTNNB*), followed by a complex formation with T-cell fate (*TCF*) to activate transcription of WNT-target genes.

It was shown in mice that one of the Wnt receptors, Fzd5, was necessary for the expression of glial cells missing homolog 1 (*Gcm1*), an important transcription factor for placental labyrinth development, followed by induction of syncytin-A expression [32]. Matsuura et al. [33] also showed that Wnt/Ctnnb signaling induces Gcm1-syncytin-A, the cell fusion pathway. Although the WNT signal is an important regulator for the expression of *ERV* during the period of invasive placental formation, whether the WNT signal induces *ERV* expression in the noninvasive bovine placenta has not been elucidated.

Unlike primate or murine species, conceptus attachment to the uterine endometrial epithelium and subsequent placentation in most ruminants do not occur soon after blastocyst formation [34]. In fact, the conceptus spends a prolonged period within the uterine lumen before developing a definitive attachment to the endometrial epithelium and subsequent formation of placental structures [35]. In the bovine species, ERVs such as *BERV-K1* [20], *bERVE-A* [21], and *syncytin-Rum1* [22] have been identified and their potential functions studied. It should be noted, however, that these *ERV*s are all from *env* regions; ERVs from other regions such as *gag* or *pol*, which could also function in ruminants' placental development, have not been identified or characterized. We hypothesized that *ERV*s of regions other than *env* would exist and function in the trophectoderm during the period of placental formation and functioning.

We searched for nucleotide structures of *ERV* origin, which were expressed in bovine conceptuses during the peri-attachment periods (the criteria are shown in Supplementary Figure S1). Using RNA-seq data, we found that one candidate gene with *gag/pol*, located on chromosome 7, was minimally expressed on day 20 (day 0 = day of estrus), when conceptus implantation to the uterine epithelium is initiated. Its expression was found to be elevated on day 22, when the placental formation is initiated, and its high expression continued until the middle of pregnancy. Expression of endogenous *ERV* gene in bovine trophoblast cells was induced by a WNT agonist, a common intracellular signaling for genes expressed in placentas.

Materials and methods
Animals and sampling
All animal procedures in the present study were approved by the Committee for Experimental Animals at Zen-noh Embryo Transfer (ET) Center, Hokkaido and the University of Tokyo, Tokyo, Japan. Estrous synchronization, super-ovulation, artificial insemination and ET processes were performed as previously described [36]. Day 7 blastocysts were collected from super-ovulated and artificially inseminated Japanese black cattle. Sixteen blastocysts derived from the super-ovulation were transferred nonsurgically into the uterine horn of eight estrous synchronized Holstein heifers ($n = 2$ blastocysts per transfer), ipsilateral to the CL on day 7. Elongated conceptuses were then collected nonsurgically by uterine flushing (500 ml PBS) on day 17 ($n = 2$), 20 ($n = 2$), or 22 ($n = 4$) [37], corresponding to the period before conceptus attachment to the uterine epithelium, when conceptus attachment is initiated, or 2 days into conceptus attachment, respectively. Days 17 and 20 conceptuses ($n = 4$ each day) in the uterine flushing media were obtained by centrifugation at $400 \times \boldsymbol{g}$ for

5 min, snap-frozen and transferred to Animal Resource Science Center at the University of Tokyo. The remaining day 22 pregnant heifers ($n = 2$) were killed and hysterectomized, from which uteri were excised and frozen immediately using the OCT compound (Sakura Finetek, Tokyo, Japan).

Hysterectomy was performed to collect days 45 and 150 pregnant uteri ($n = 3$ each) from Japanese black cattle at Azabu University Veterinary Teaching Hospital (AUVTH), Kanagawa, Japan and the National Institute of Agrobiological Sciences (NIAS), Ibaraki, Japan, respectively, from which cotyledons and caruncles were dissected, immediately frozen and stored at −80°C until use. At NIAS, various tissues, heart, liver, kidney, intestine, lung, muscle, skin, lymph node, and spleen were harvested from day 150 pregnant Japanese black cattle, and the uteri were removed from three nonpregnant Japanese black cattle. All tissues dissected were frozen immediately and stored at −80°C until use.

RNA extraction from bovine conceptus and whole-genome sequencing by the RNA-seq (SOLiD3) system

RNA extraction from whole conceptus tissues was performed using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol [38]. For a RNA-seq analysis, total RNA was depleted of rRNAs using the RiboMinus Eukaryote Kit (Life Technologies, Carlsbad, CA, U.S.A.). Details of the sequencing procedure using a SOLiD3 sequencer (Life Technologies), and data analysis have been described previously [37]. In brief, high-throughput sequencing libraries were prepared according to the SOLiD3 whole transcriptome library preparation protocol [39]. *ERV*-derived sequences in the bovine genome (Btau_4.0.55.dna.toplevel.fa) were identified through the use of RetroTector, which was designed to detect and characterize entire or fragmented *ERV*s in a given genome [40]. Four types of retroviral-like sequences identified in the genome are *gag*, *pro*, *pol*, and *env*. Processed nucleotide sequences from the RNA-seq for each of the 3 days examined were aligned against the bovine genome. The Applied Biosystems Whole Transcriptome Analysis Pipeline was used to map short reads. During this mapping phase, up to two mismatches were allowed and short reads that aligned to more than 10 locations were removed. In this analysis, we used the reads whose sequence quality scores were 24 or higher, following the standard parameters of the Applied Biosystems Whole Transcriptome Analysis Pipeline [39]. Matching locations were subsequently used to generate counts for identified ERVs and Ensemble-provided coding sequences (Bos_taurus.Btau_4.0.55.dna.toplevel.fa). To evaluate gene expression level independent of variance in gene lengths and the number of reads among samples, the method of reads per kilobase of exon model per million mapped reads (RPKM), a widely recognized quantification measurement, was applied [41]. The RNA-seq data can be downloaded from the DDBJ Sequence Read Archive [42] with accession number DRA000549 [37].

Cell culture

All cells were maintained at 38.5°C in humidified 5% CO_2 and cultured up to confluence. Bovine trophoblast BT-1 [43], CT-1 [44], and F3 [45] cells were kindly provided by Prof. K. Hashizume (Iwate University, Japan), Prof. A. Ealy (Virginia Polytech Institute, U.S.A.), and Prof. C. Pfarrer (University of Veterinary Medicine, Hannover, Germany), respectively. BT-1 cells were cultured on plastic plates coated with type I collagen (Nitta Gelatin, Osaka, Japan) in Dulbecco's modified Eagle medium (DMEM)/Ham's F12 medium (F12) (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, U.S.A.) and antibiotic/antimycotic solution (Invitrogen). CT-1 cells were maintained in DMEM (Invitrogen) containing 10% (v/v) FBS (JRH Biosciences) supplemented with 4.5 g/l D-glucose, nonessential amino acids, 2 mM glutamine, 2 mM sodium pyruvate, 55 mM β-mercaptoethanol, and antibiotic/antimycotic solution (Invitrogen). F3 cells were cultured in DMEM/F12 medium supplemented with 10% FBS (JRH Biosciences) and antibiotic/antimycotic solution (Invitrogen). Bovine primary uterine endometrial epithelial cells (EECs) and stromal cells (STRs) were kindly provided by Prof. K. Okuda (Obihiro University, Japan) and were cultured in DMEM/F12 medium (Invitrogen) supplemented with 5% FBS (JRH Biosciences) and antibiotic/antimycotic solution. Bovine intestinal epithelial cells (Bie) were kindly provided by Prof. H. Aso (Tohoku University, Japan) and were maintained in DMEM/F12 (Invitrogen) supplemented with 10% FBS (JRH Biosciences) and antibiotic/antimycotic solution. Bovine ovarian cumulus-granulosa (oCG) cells were obtained from ovarian follicles that had been collected at a local abattoir, and ear-derived fibroblast (EF) cells were obtained from biopsied ear skin of Japanese black bulls (4 months old) [38], both cells were cultured in DMEM (Invitrogen) containing 5% (v/v) FBS (JRH Biosciences) and antibiotic/antimycotic solution. Bovine kidney cell lines

(MDBK and CKT-1) and bovine macrophage cells (BoMAC) were cultured in DMEM (Invitrogen) supplemented with 10% v/v FBS (JRH Biosciences) and antibiotic/antimycotic solution. To study the effects of trophoblast attachment to the uterine endometrial epithelial cells, CT-1 cells were cultured without or with a cell culture insert (Falcon, BD Biosciences, Tokyo, Japan), allowing direct CT-1 cell contact to EECs or indirect cell association with EECs, respectively. To further characterize whether any of the candidate *ERV* genes could be regulated by Wnt signaling, cultured CT-1 or F3 cells were treated with 1 μM Wnt agonist (sc-222416, Santa Cruz Biotechnology, Dallas, TX, U.S.A.) for 24 h.

RNA isolation from bovine tissues and cultured cells

RNA isolation from bovine tissues and cultured cells was performed using the ISOGEN protocol (Nippon Gene), as described previously [38]. Bovine tissues, heart, liver, kidney, intestine, lung, muscle, skin, lymph node, spleen, and uterus were harvested from three Japanese black cattle at NIAS, Ibaraki, Japan. Excised tissues were submerged in RNAlater (Qiagen, Tokyo, Japan) to prevent RNA degradation, and RNA was then extracted from each tissue. RNA was also isolated from bovine cell lines, including trophoblast cell lines (BT-1, CT-1, and F3), EEC, STR, CKT-1, MDBK, Bie, EF, oCG, and BoMAC. Extracted RNAs were then stored at −30°C until use.

PCR analysis

For PCR and real-time PCR analyses of conceptus RNA, isolated RNA (total 0.5 μg) was reverse-transcribed to cDNA using the ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) in a 10 μl reaction volume, and the resulting cDNA (RT template) was stored at 4°C until use. The cDNA reaction mixture was diluted 1 : 10 using DNase-, RNase-free molecular biology grade water. RT template (cDNA) was subjected to PCR or real-time PCR amplification using specific primers (Table 1). PCR-amplified products were separated on 1.5% (w/v) agarose gels after 32 cycles, from which PCR products were subcloned and verified by DNA sequencing. Quantitative PCRs were performed using the SYBR Green kit (Takara Biomedicals, Tokyo, Japan) and the Applied Biosystems thermal cycle system (7900HT, Applied Biosystems, Tokyo, Japan), as previously described [38]. Real-time PCR was performed under the following thermal cycling conditions: 10 min at 95°C, and 40 cycles of 95°C for 10 s followed by 60°C for 30 s. Average cycle threshold (C_t) values for all mRNAs examined were calculated and normalized to C_t values for *ACTB* mRNA.

RNA isolation from bovine conceptus tissues and 5′-RACE for the characterization of the 5′-side of a full-length *BERV-K3* transcript

Total RNA was extracted from day 22 bovine conceptuses using the RNeasy Mini Kit together with the RNase-free DNase Set (Qiagen). To identify a full-length *BERV-K3* transcript, 5′-RACE with the primer (P1R prime, Table 1 and Supplementary Figure S2) was used to synthesize a first-strand cDNA using the SMARTer RACE 5′/3′ kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. PCR was performed using PrimeSTAR Max (Takara) under the following thermal cycling conditions: 5 cycles of 98°C for 10 s followed by 72°C for 90 s, 5 cycles of 98°C for 10 s followed by 68°C for 90 s, 30 cycles of 98°C for 10 s, 55°C for 10 s followed by 72°C for 90 s, and 1 cycle of 72°C for 10 min. The PCR product was separated by agarose gel electrophoresis and then visualized with a UV trans-illuminator, from which PCR products were subcloned and verified by DNA sequencing.

In situ hybridization

Frozen day 22 uteri embedded in OCT compound were sectioned (10 μm), fixed in 10% neutral buffered formalin, and then subjected to hybridization. Various bovine tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μm), and mounted on silane-coated slides (Zyagen, San Diego, CA, U.S.A.). Slide sections were blocked with Block Ace at room temperature for 1 h, incubated with DIG-labeled antisense or sense riboprobe, and then prepared using the *BERV-K3* fragment (nucleotide 150 bases) in the pGEM-T Easy Vector with T7 and SP6 promoters (Promega, Madison, WI, U.S.A.) and an RNA transcription kit (Toyobo, Tokyo, Japan) according to the manufacturer's protocol. As described previously [46], hybridization was performed in a humidified chamber at 42°C for 18 h, and bound probes were visualized using alkaline phosphatase-conjugated anti-DIG antibody with nitro blue tetrazolium and 5-brome-4-chloro-3-indolyl phosphate (Promega) as substrates.

PORTLAND
PRESS

Table 1 Primer sets for _BERV-K3_ and neighboring gene transcripts

BERV-K3 (P1 in Figure 1B)

For detection of the transcript and preparation of probes for _in situ_ hybridization

    P1F: 5′-TCGCCCGAAAGCAGGCTAGTGCTAA-3′

    P1R: 5′-CAAGGGCGCAGGCTGTTACCTGTTC-3′

For confirming if _BERV-K3_ is expressed (in Supplementary Figure S3A)

    P1F: 5′-TCGCCCGAAAGCAGGCTAGTGCTAA-3′

    P1R′: 5′-GCAAGGTTCCGTTTTATGG-3′

For 5′-RACE primer (in Supplementary Figure S3B)

    P1R: 5′-CAAGGGCGCAGGCTGTTACCTGTTC-3′

For isolated predicted full length (in Supplementary Figure S3C)

    PFF: 5′-ACGGGTAACAAGGAGTCAAAAG-3′

    PFR: 5′-CCCTGATGACAAAGTGACCTCC-3′

The primer set to detect _LOC100848658_ (P2 in Figure 1B) transcript

    F: 5′- GCGTCTACCCCAAAACCAGA-3′

    R: 5′- ACAGAGAAAGGTGGTCAGGG-3′

The primer set to detect _TCF7_ (NM_001099186.2)

    F: 5′- CTGTGAGCTGGTTCACCCAT-3′

    R: 5′- TCCGCAATGACTTTGGCTCT-3′

The primer set to detect _ACTB_ (NM_173979.3)

    F: 5′-CTCTTCCAGCCTTCCTTCCT-3′

    R: 5′-GGGCAGTGATCTCTTTCTGC-3′

F, forward primer; R, reverse primer.

Statistical analysis

Quantitative data were subjected to one-way analysis of variance using the Statistical Analyses System (SAS Institute, Cary, NC, U.S.A.); the SEMs shown in the figures were derived from this. When a significant effect of day of pregnancy was detected ($P < 0.05$), the data were analyzed by Dunnett's test. In these analyses, day of pregnancy was considered an independent source of variation, and replicate was a dependent source.

Table 2 The listing of candidate 10 retroelements detected

Transcript ID	Origin	Tag numbers			RPKM			Length	Chr.	Strand
		D17	D20	D22	D17	D20	D22			
ENSBTAT00000012578	Znf	89	105	54	10.814	8.406	5.539	707	2	–
ENSBTAT00000052900	Gag	9	3	0	0.312	0.069	0	2472	4	–
ENSBTAT00000057217	Gag	407	7	0	18.440	0.209	0	1896	6	–
ENSBTAT00000053802	Gag	435	11	0	19.462	0.324	0	1920	6	–
ENSBTAT00000007724	Env	8	6	0	0.376	0.186	0	1827	6	–
ENSBTAT00000052494	Gag	6	1	44	0.318	0.035	1.973	1617	7	+
ENSBTAT00000052446	Gag	7	1	56	0.244	0.030	1.649	2463	7	+
ENSBTAT00000045982	Env	2	1	0	0.102	0.034	0	1680	11	–
ENSBTAT00000056638	Gag	2	4	0	0.070	0.092	0	2469	24	+
ENSBTAT00000029917	Env	157	14	0	6.801	0.400	0	1983	X	–

RPKM, reads per kilo base-pair per million mapped reads; Chr., chromosome.

Results

Searching for *ERV*-derived elements expressed during the peri-attachment period

We experimentally validated that the expression of *ERV*-derived sequences that met all of the following criteria: (1) was identified as an *ERV*-derived sequence from the bovine genome (Btau4.0) by RetroTector, possessing an ORF at least 100 amino acids or has at least one transmembrane domain, (2) was overlapped or located near or in between transcript regions annotated in the Ensembl database (http://www.ensembl.org), allowing two mismatches and annotated more than 10 multiloci, and (3) was detected on at least 2 days (day 17, 20, or 22) by the SOLiD3 sequencing (Supplementary Figure S1). Using these criteria, 10 putative *ERV*s that are located in between functional genes of the bovine genome were identified (Table 2 and Supplementary Table S1), after which another criterion, up-regulation of transcripts on day 22, was imposed in the analysis. Although the expression of 7 out of 10 putative *ERV*s declined, the expression of three candidate transcripts was up-regulated in day 20 or 22 pregnant conceptuses. Two transcripts, that both exist on chromosome 7 (ENSBTAT00000052494 and ENSBTAT00000052446, respectively), increased on day 22 (Table 2). In addition, transcript of a third candidate that exists on chromosome 2 (ENSBTAT00000012578) was detected on day 20 when trophoblast attachment to uterine endometrial epithelial cells was initiated, but not on day 22, and was excluded from the subsequent studies.

Identification of candidate *ERV* sequence and neighboring genes and search for its *LTR*-like sequences

Using *in silico* analysis, we re-annotated the equivalent region on chromosome 7 with the recent database, *Bos Taurus* UMD3.1 version; however, the candidate *ERV*s previously identified were not detected. PCR analysis subsequently carried out using the primers (P1, Figure 1B) identified a portion of the *ERV* transcript, ENSBTAT00000052446 (Btau4.0) predicted from the original RNA-seq analysis, from which the sequences were extended through the use of RACE method, resulting in the identification of one *ERV* sequence (Supplementary Figure S2) existed on chromosome 7 as shown in Figure 1A. Subsequently, we identified the *ERV* mRNA on chromosome 7, containing *Gag*_p10, *Gag*_p24, and zinc finger (ZnF_CCHC) motifs, and short introns in between ORF 1 and 2 (2 nt) and between ORF 2 and 3 (36 nt) as shown in Figure 1B and Supplementary Figure S2. The analysis was extended to determine whether actual transcripts existed in the bovine conceptuses, and found that the single *ERV* transcript was, in fact, amplified by RT-PCR (Supplementary Figure S3A). The amino acid sequence of the *ERV* transcript was then used as a query against an ERV ORF database called gEVE [47], identifying that the *ERV* sequence was quite similar to the *gag* sequence of *BERV-K2* [20] (Supplementary Figure S4A). Because the presence of LTRs (long terminal repeats) or LTR-like sequences was expected, attempts were made to identify the presence of LTR or LTR-like sequences on the chromosomal regions between 20 kb upstream of and 20 kb downstream from the *ERV* sequence. Two 988 and 1036 nt alignable sequences, located −333 nt upstream of and 6097 nt downstream from the ERV, respectively, were found, of which sequences were also similar to those of *BERV-K2* (Supplementary Figure S4B). We, therefore, named the putative ERV transcript as *BERV-K3*.

 BERV-K3 was located between interleukin enhancer-binding factor 3 (*ILF3*) and *QTRT1* genes, and *LOC100848658* was also found as a gene that shares a partial sequence (Figure 1A,B). The *BERV-K3* sequence was then expected to be located on equivalent regions of chromosomes in human, murine, and sheep genomes; however, *BERV-K3* sequence was not found in these species. These data indicated that the *BERV-K3* is unique to the bovine species. In some cases, if these LTRs harbored appropriate binding sites for trophoblast-specific transcription factors, they may function as active promoters or enhancers with the potential to modulate neighboring gene expression [48]. We, therefore, examined the expression of *ILF3* and *QTRT1* with SOLiD3 data (Table 3); however, these gene expressions were not parallel to that of *BERV-K3* expression.

Expression of *ERV*-derived transcripts in bovine conceptuses and other tissues

Using real-time PCR analysis, the expression of candidate transcripts was found in days 17, 20, and 22 bovine conceptuses and days 45 and 150 cotyledons. In particular, the expression of *BERV-K3* was detected on day 20, when conceptuses begin to attach to uterine epithelial cells, in agreement with RNA-seq and real-time PCR
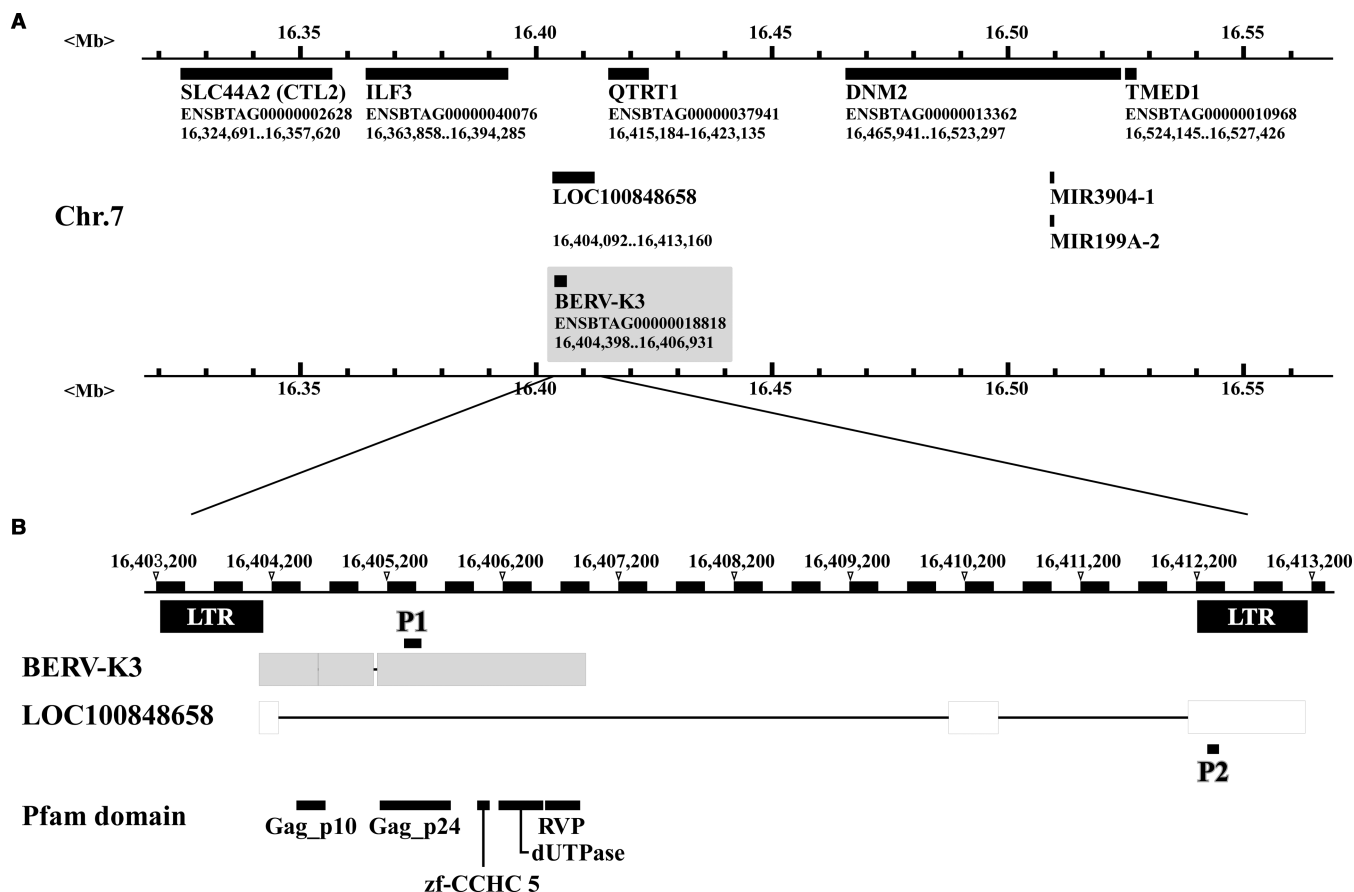
Figure 1. Identification of *BERV-K3* and their neighboring genes on chromosome 7 of the bovine genome.
(**A**) Genes neighboring *BERV-K3*. Solid-black bar indicates the location of nucleotide sequences and their identity is given below the solid-black bar. Black bars within the gray box is *BERV-K3*. (**B**) Structures of *BERV-K3*, Pfam domains, and primers set to detect transcripts. P1, indicated by a short-black line, is the primer set 1, which detects *BERV-K3*. P2, indicated by a short-black line, is the primer to detect *LOC100848658*. Pfam domains (*Gag*_p10, *Gag*_p24, zf-CCHC5, dUTPase and RVP) relative to *BERV-K3* structures are given below.

analyses (Figure 2A). *LOC100848658* gene was similarly expressed with *BERV-K3* in bovine conceptuses, but not in other tissues examined (Figure 2 and Supplementary Figure S5). *BERV-K3* was highly expressed in coty-ledon on days 45 and 150 pregnancy, and either expression levels were similar to that in day 22 conceptuses. The expression of *BERV-K3* in various bovine tissues was then examined (Figure 2B) and found that *BERV-K3* was expressed in muscle, uterus, and skin. The expression of *BERV-K3* in various bovine cell lines was further

Table 3 The Tag numbers and RPKM values of *BERV-K3* neighboring genes.

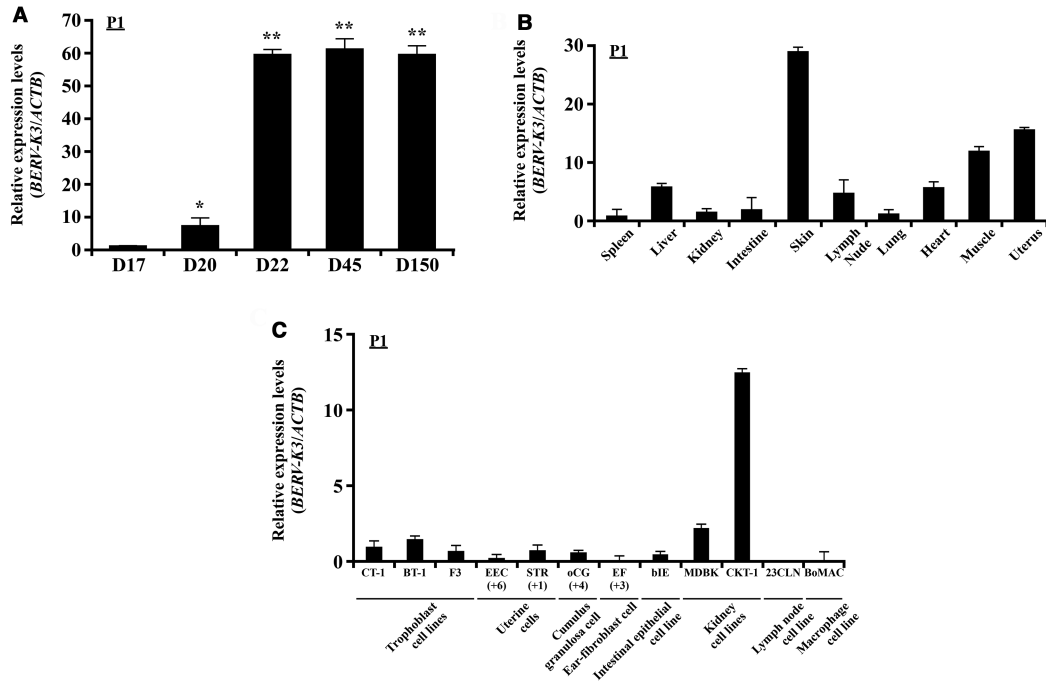| Transcript ID	Gene name	Tag numbers			RPKM		
		D17	D20	D22	D17	D20	D22
ENSBTAT00000003403	SLC44A2 (CTL2)	6650	11 253	2454	175.50	195.68	54.67
ENSBTAT00000040076	ILF3	1360	1220	1036	36.04	21.30	23.17
ENSBTAT00000056840	QTRT1	71	28	47	11.25	2.92	6.28
ENSBTAT00000036121	DNM2	984	853	627	24.52	14.00	13.18
ENSBTAT00000014563	TMED1	268	310	416	15.33	11.68	20.08

Figure 2. Expression of *BERV-K3* transcript in bovine conceptuses and various tissues and cell lines.

(**A**) Changes in *BERV-K3* expression. Using primer set P1 described in Figure 1B, expression levels of transcript were determined in day 17, 20, and 22 (D17, D20, and D22) conceptuses ($n = 4$, each day) and day 45 and 150 cotyledons ($n = 3$, each day). RNAs from four or three of conceptuses or cotyledons, respectively, were used to perform the qPCR analysis. Data are expressed as fold difference relative to its expression on D17. *, **Statistically significant differences in mRNA levels ($P < 0.05$ or $P < 0.01$, respectively) when compared with that in D17. (**B**) *BERV-K3* expression in various tissues. *BERV-K3* transcripts in various tissues of day 150 pregnant animals, except that uterine sample was harvested from nonpregnant Japanese black cattle. RNAs were extracted from the spleen, liver, kidney, intestine, skin, lymph node, lung, heart, muscle, and uterus (cyclic) ($n = 3$ each). Muscle and skin were taken from the upper left hind leg area. Data are expressed as fold difference relative to the expression in the spleen. (**C**) *BERV-K3* expression in bovine cell lines. *BERV-K3* transcripts in cultured bovine primary cells and cell lines. CT-1, BT-1, and F3 are bovine trophoblast cell lines. Primary uterine cells (EPI and STR; indicated passage times below), primary cumulus-granulosa cell (oCG; indicated passage times below), primary ear-fibroblast cell (EF; indicated passage times below), intestinal epithelial cell line (bIE), kidney cell lines (MDBK and CKT-1), lymph node cell line (23CLN), and macrophage cell line (BoMAC) were subjected to the present study. Three samples each were used and three independent experiments were conducted. Data are expressed as fold difference relative to the expression in CT-1.

examined (Figure 2C) and found that although some expressions were detected in bovine trophoblast cell lines, *BERV-K3* was highly expressed in bovine kidney CKT-1 cells.

In situ localization of *BERV-K3* transcript in the uterus and other tissues

BERV-K3 transcript was found in day 22 uteri, including conceptus, uterine epithelial, and glandular epithelial cells (Figure 3). In addition, *BERV-K3* transcript was found in the skin, liver, kidney, and intestine (Figure 4). In the skin, *BERV-K3* was localized in outer root and inner root sheath (ORS and IRS, respectively) areas of hair follicles and the stratum spinosum of epidermis. In the liver, *BERV-K3* was detected in hepatic laminas of lobule areas, particularly in hepatocytes surrounding central and interlobular veins (ILVs), and in interlobular bile ducts (ILB) of portal canal areas. In the kidney, *BERV-K3* transcript was found in epithelial cells of renal-collecting ducts and distal convoluted tubules, and in infiltrating lymphocytes around renal corpuscle; however, *BERV-K3* transcript was not found in the cortex region. In the ileum, *BERV-K3* was localized in intestinal epithelia and enterocytes in glandules, and in Peyer's patches region. Positive staining was also detected on lymphocytes located in ileum lymphoid follicles and in infiltrating lymphocytes near renal corpuscles. These results indicated that the expression of *BERV-K3* was not restricted to the reproductive tissues.
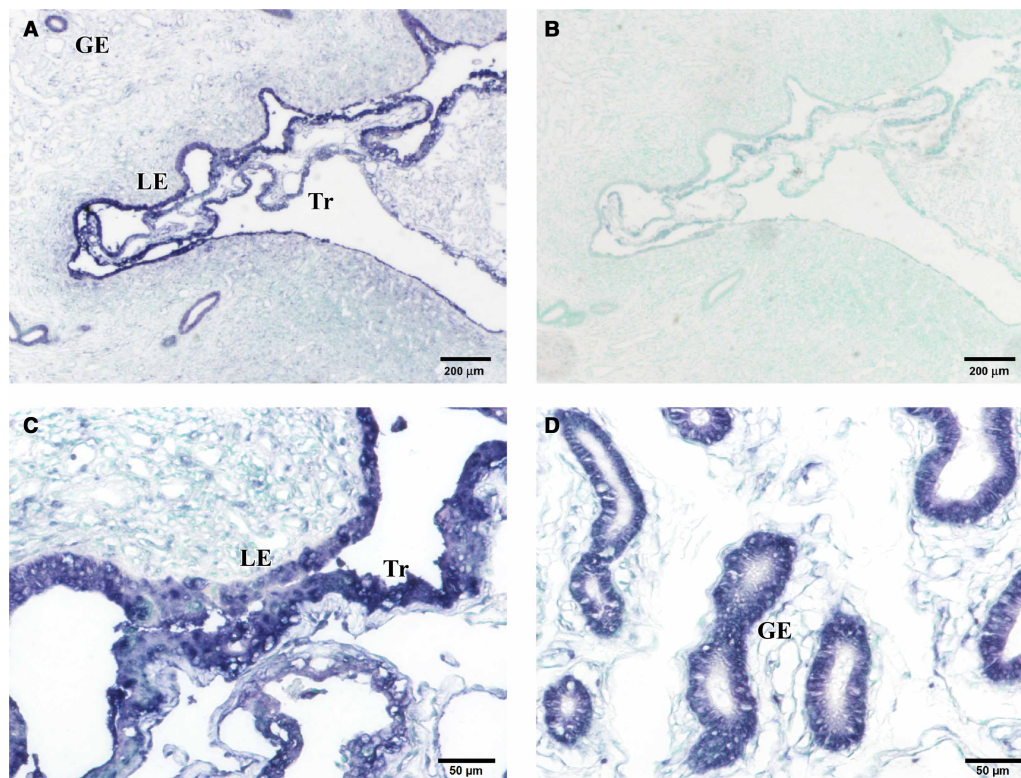
Figure 3. *In situ* localization of *BERV-K3* transcript in day 22 pregnant uteri.
(**A**) *In situ* localization of *BERV-K3* transcript in day 22 pregnant animals. (**B**) *In situ* localization with the sense-strand probe (negative control). (**C**) Higher magnification showing luminal epithelia and elongated trophoblasts. (**D**) Higher magnification showing glandular epithelial regions. GE, glandular epithelia; LE, luminal epithelia; Tr, trophoblast.

Induction of *BERV-K3* transcript by cell-to-cell contact or canonical WNT agonist

When CT-1 cells were cultured with cell culture insert, not allowing direct CT-1 contact to EECs, no increase in *BERV-K3* transcripts was found. However, when CT-1 cells were cultured without the cell insert, allowing direct cell-to-cell contact, increase in *BERV-K3* was found (Figure 5A). Because WNT signal is known to be important for placentation following conceptus attachment to the uterine epithelium in the bovine species [29] and our previous study [37] showed that *WNT2B* and its receptor *FZDs* mRNA were detected during the conceptus attachment period, we then treated trophoblast CT-1 and F3 cells with 1 μM of canonical WNT agonist for 24 h. *BERV-K3* and *TCF7*, but not *LOC100848658*, were induced by the WNT agonist in both cells (Figure 5B and Supplementary Figure S6).

Discussion

In the present study, we found *ERV*, putative *gag/pol*-derived *BERV-K3*, transcripts specifically expressed in the bovine placenta from early- to mid-gestation. Trophectoderm and fetal membranes had weak expression of *BERV-K3* transcript on day 20 and had greater expression from days 22 to 150. Accordingly, the transcripts were detected in bovine trophoblast CT-1, BT-1 and F3 cells. However, the transcripts were also found in the uterus, skin, liver, kidney, and ileum, indicating that their expression appeared somewhat ubiquitous. Cornelis et al. [22] similarly reported that the *Bos-Env2* was expressed in the skin, spleen, and muscle, whereas only limited expression was detected in the bovine placenta. To elucidate molecular mechanisms associated with *BERV-K3* transcription, the co-culture system with CT-1 cells and EECs [49] was used to study difference in *BERV-K3* expression between days 20 and 22, when bovine conceptus begins its attachment to the uterine epithelium cells on day 20, followed by the tight attachment between two cell types on day 22. When CT-1 cells were cultured
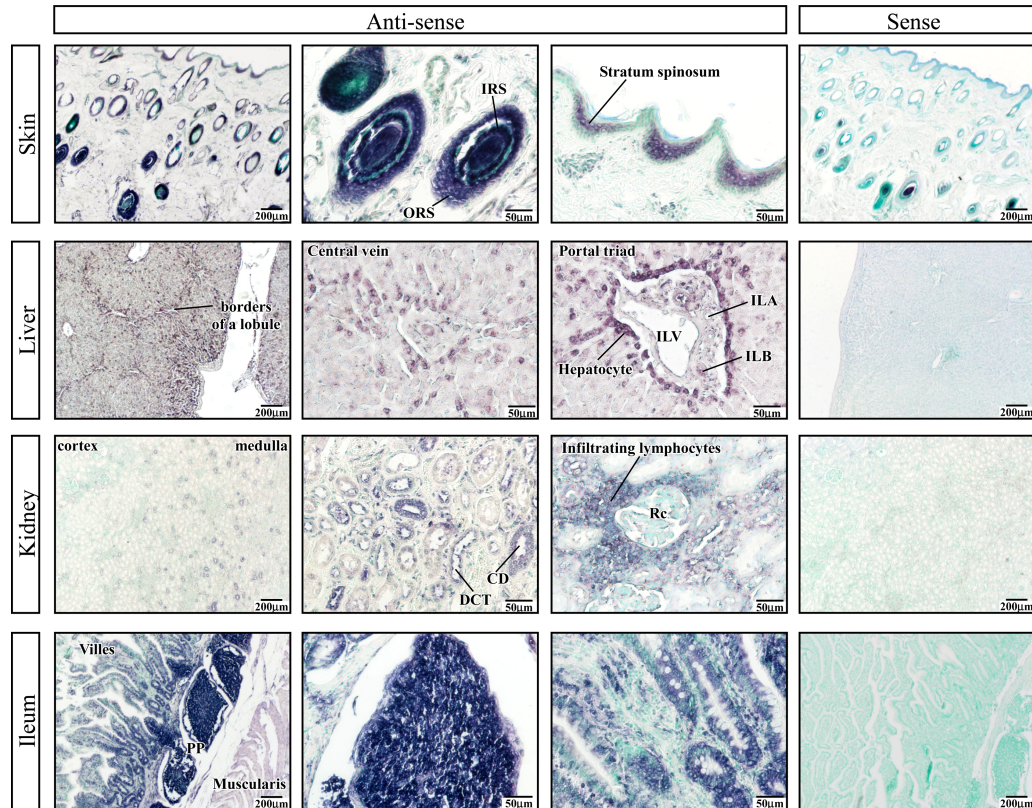
Figure 4. *In situ* localization of *BERV-K3* transcript in the skin, liver, kidney, and ileum.
Bovine tissue sections were purchased from Zymogen (San Diego, CA, U.S.A.). In the skin, *BERV-K3* transcript is found in external root and internal root sheath areas of hair follicles and stratum spinosum of epidermis. ORS, outer root sheath; IRS, inner root sheath. In the liver, *BERV-K3* is seen in hepatic laminas of lobule areas, particularly in hepatocytes surrounding central veins and ILV, and in interlobular bile ducts of portal canal areas. ILA, interlobular artery; ILB, interlobular bile duct; ILV, interlobular vein. In the kidney, *BERV-K3* transcript is found in epithelial cells of renal-collecting ducts and distal convoluted tubules, and in infiltrating lymphocytes around renal-collecting ducts. CD, collecting duct; DCT, distal convoluted tubule; Rc, renal corpuscle. In the ileum, *BERV-K3* is seen in intestinal epithelia and enterocytes in glandules, and in PP regions. PP, Peyer's patch. In each, the far right images represent those with the sense-strand cRNA probe.

without the cell culture insert, increase in *BERV-K3* transcripts was observed, suggesting that the cell-to-cell contact of trophoblasts to the uterine epithelial cells is required for *BERV-K3* transcription to increase.

In our *in silico* analysis, LTRs (ERVK-type) were found to be located on both upstream and downstream regions of the *BERV-K3* gene. However, while *BERV-K3* was expressed in the trophectoderm and uterus, additional expression in the skin, liver, kidney, and ileum indicates that these LTRs are not pregnancy-specific. Transcripts of *syncytin-Rum1*, a fusogenic protein required for ruminants' placentation, were also detected in maternal (caruncle and intercarunclar endometrium) and fetal membranes (chorioallantois) [50]. Several genes are located in close proximity to *BERV-K3* on chromosome 7 of the bovine genome, including *ILF3* and *CTL2* (also known as SLC44A2), located on the upstream region, and *DNM2* and *TMED*, located on the downstream region of the *BERV-K3* locus. Increase in *BERV-K3* transcripts in day 22 bovine conceptuses was initially considered trophoblast/placenta-specific; however, the expression of these transcripts other than in the trophectoderm indicates that the insertion of *BERV-K3* did not cause the placenta-specific expression of *TMED* and *CTL2*. It should be noted that although human and murine genomes lack *BERV-K3*, *TMED*, and *CTL2* are expressed in placentas of these species. In fact, TMED2/p24b1 protein is required for the morphogenesis of the mouse embryo and placental development [51]. The expression of *CTL2* transcripts in bovine conceptuses was high, peaking on day 20 when the conceptus begins its attachment to the uterine endometrial epithelium.
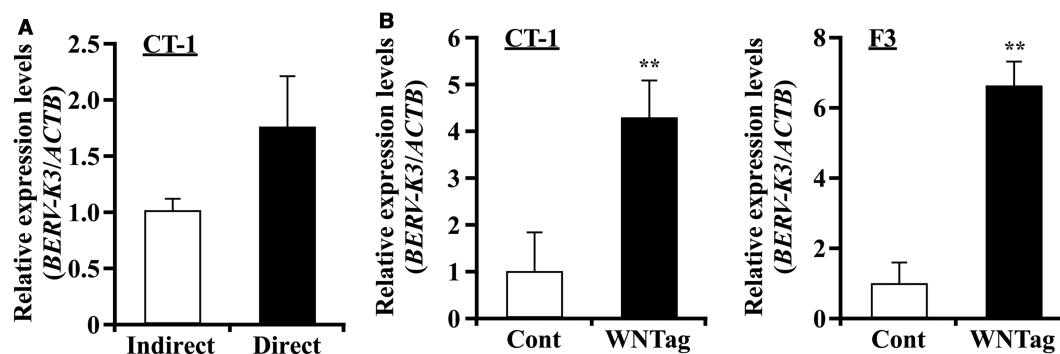
Figure 5. Induction of *BERV-K3* expression in CT-1 by cell-to-cell contact or in CT-1 or F3 cells by treatment with the canonical WNT agonist.

(**A**) Cell-to-cell contact on *BERV-K3* expression. CT-1 cells were co-cultured without or with a cell culture insert on EECs for 24 h. (**B**) Effect of WNT agonist on *BERV-K3* expression. CT-1 (left) or F3 (right) cells were treated with 1 μM of canonical WNT agonist for 24 h. Data are expressed as fold difference relative to the expression in control. **Statistically significant differences in mRNA levels ($P < 0.05$) when compared with that in control.

Rather than the insertion of *BERV-K3* in between *TMED* and *CTL2* genes affected the transcriptional regulation of these genes, it is probable that these genes may play a role in the regulation of *BERV-K3* gene expression in bovine trophoblasts. Although viral integration to the host genome could occur in a random manner, the integration would have to be locus-specific, if the integrated gene was to become active in certain cell types and/or physiological conditions.

WNT agonist used in the present study as a WNT signal activator was found to induce endogenous *BERV-K3* expression in bovine trophoblast CT-1 and F3 cells. The WNT signaling pathway has been established as an important regulator of embryo/conceptus implantation and placental development in mice, sheep, cow, and humans [27–30]. Our previous study in peri-attachment conceptuses revealed that *WNT2B* and its receptor 9 *FZD*s mRNA were detected, while *DKK-1*, which acts as an antagonist of the canonical WNT signaling pathway, was decreased in day 22 bovine conceptuses [37]. Although an experiment determining the effect and function of DKK-1 is beyond the scope of the present study, it is possible that the decrease in the *DKK-1* expression after trophoblast attachment to the uterine epithelial cells could lead to activation of canonical WNT signaling. In response to activation of *CTNNB* and *TCF*-dependent transcription by the Wnt agonist, *BERV-K3* mRNA was increased in trophoblast CT-1 and F3 cells, suggesting that *BERV-K3* could be one of the target genes in the canonical WNT pathway. It was also found that epithelial–mesenchymal transition (EMT) occurs in day 22 bovine conceptuses [52,53]. Because the WNT signal is involved in the EMT process, together with WNT, BERV-K3 may play a role in the bovine conceptus EMT and/or its attachment to the uterine epithelium.

Although envelope proteins of *HERV*s expressed in human trophoblast cells have fusogenic activity and immunosuppressive properties [9,54], the roles that gag polyproteins play have not been definitively elucidated. Most prominent *gag*- or *gag/pol*-derived gene is *PEG10*, which is thought to have been derived from the Ty3/Gypsy family of retrotransposons and is expressed as two proteins: *gag*-like protein containing a CCHC-type zinc finger domain and *gag/pol*-like fusion protein with an additional aspartic protease motif [55,56]. The *Peg10* knockout mice showed early embryonic lethality due to defects in placental formation [57]. Although the amino acid sequences differ substantially between PEG10 and BERV-K3 (data not shown), BERV-K3 is also a *gag/pol*-derived ERV and has similar motifs to those of PEG10. *BERV-K3* was found to consist of *Gag*_p24 and *Gag*_p10 motifs: Gag_p24 forms inner protein layer of the nucleocapsid, while Gag_p10, a retroviral GAG (core) protein, encompasses the p10 region producing the p10 protein upon proteolytic cleavage of GAG by retroviral protease. The p10 is, in fact, associated with viral envelope glycoproteins in most mammalian retroviruses and may be involved in viral particle assembly, transport, and budding [58]. *BERV-K3* also consists of the motifs that potentially encode structural proteins, including zinc finger domain, UTPase, and retroviral aspartyl protease; however, the function of these motifs in BERV-K3 has not been elucidated. Nevertheless, because of its insertion in this specific locus, BERV-K3, unique to the bovine species,

could have acquired its capability for expression and/or possibly functioning together with neighboring genes in the bovine placenta.

In the present study, *BERV-K3* was also detected in IRS, ORS, and stratum spinosum in skins. It was found that the Wnt/Ctnnb signaling system was detected to function in mouse IRS and ORS [59]. In addition, retroviral-like aspartic protease (SASPase) is expressed in the stratum granulosum of mouse skin and is involved in wrinkle formation and adjustments of the stratum corneum hydration [60,61]. *BERV-K3* transcripts found in skin epidermis suggest that BERV-K3 protein could be involved in skin development, although further experimentation is required to characterize its molecular mechanism associated with these events. In the liver, *BERV-K3* was detected in hepatocytes surrounding central vein and ILV, in which the Wnt/Ctnnb was detected in pericentral hepatocytes in mice [62]. Involvement of canonical Wnt signaling in intestinal epithelial cell proliferation has also been demonstrated [63]. These and results from the present study suggest that in addition to placental formation and its maintenance, BERV-K3 may play a role in the development and/or maintenance of skin, liver, and intestines.

In conclusion, we found the new *gag/pol*-derived *ERV* transcript in RNAs extracted from days 20 to 22 bovine conceptuses, of which *ERV* was integrated into the chromosome 7. The expression of *BERV-K3* gene increased in bovine conceptuses following its attachment to uterine epithelia and its high expression was maintained in the placenta up to 150-day gestation. Although the function of the genes is yet to be elucidated, available data suggest that *BERV-K3*, unique to the bovine species, could be controlled initially through cell-to-cell contact and then by WNT signaling and plays developmental and/or maintenance roles in placenta, skin, liver, and intestines.

Abbreviations

BERVs, bovine endogenous retroviruses; Bie, bovine intestinal epithelial cells; *CTNNB*, cellular accumulation and nuclear translocation of β-catenin; DMEM, Dulbecco's modified Eagle medium; EECs, endometrial epithelial cells; EF, ear-derived fibroblast; EMT, epithelial–mesenchymal transition; ERVs, endogenous retroviruses; FBS, fetal bovine serum; *FZD*, Frizzled; *Gcm1*, glial cells missing homolog 1; ILB, interlobular bile duct; *ILF3*, interleukin enhancer-binding factor 3; ILV, interlobular vein; LTRs, long terminal repeats; oCG, ovarian cumulus-granulosa; ORFs, open-reading frames; ORS and IRS, outer root and inner root sheath; RPKM, reads per kilobase of exon model per million mapped reads; STRs, stromal cells; *TCF*, T-cell fate.

Author Contribution

J.Y., T.M., and K.I. co-ordinated the project. S.N. performed analyses related to bioinformatics. T.S., H.B., R.B., K.K., A.I., Y.A., K.K., and K.I. equally performed the experiments. T.S. and K.I. wrote the manuscript, but all authors were involved in discussion throughout the experimentation and manuscript preparation. All authors thoroughly read and approved the manuscript.

Funding

Acknowledgements

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

References

1 Weiss, R.A. (2006) The discovery of endogenous retroviruses. *Retrovirology* **3**, 67 https://doi.org/10.1186/1742-4690-3-67

2 International Human Genome Sequencing Consortium (2004) Finishing the euchromatic sequence of the human genome. *Nature* **431**, 931–945 https://doi.org/10.1038/nature03001

3 Chinwalla, A.T., Cook, L.L., Delehaunty, K.D., Fewell1, G.A., Fulton, L.A., Fulton, R.S. et al. (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* **420**, 520–562 https://doi.org/10.1038/nature01262

4 Adelson, D.L., Raison, J.M. and Edgar, R.C. (2009) Characterization and distribution of retrotransposons and simple sequence repeats in the bovine genome. *Proc. Natl Acad. Sci. U.S.A.* **106**, 12855–12860 https://doi.org/10.1073/pnas.0901282106

5 Boeke, J.D. and Stoye, J.P. (1997) Retrotransposons, endogenous retroviruses, and the evolution of retroelements. In *Retroviruses* (Coffin, J.M., Hughes, S.H. and Varmus, H.E., eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

6 Mi, S., Lee, X., Li, X.-p., Veldman, G.M., Finnerty, H., Racie, L. et al. (2000) Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* **403**, 785–789 https://doi.org/10.1038/35001608

7 Blaise, S., de Parseval, N., Bénit, L. and Heidmann, T. (2003) Genomewide screening for fusogenic human endogenous retrovirus envelopes identifies syncytin 2, a gene conserved on primate evolution. *Proc. Natl Acad. Sci. U.S.A.* **100**, 13013–13018 https://doi.org/10.1073/pnas.2132646100

8 Kudaka, W., Oda, T., Jinno, Y., Yoshimi, N. and Aoki, Y. (2008) Cellular localization of placenta-specific human endogenous retrovirus (HERV) transcripts and their possible implication in pregnancy-induced hypertension. *Placenta* **29**, 282–289 https://doi.org/10.1016/j.placenta.2007.11.009

9 Esnault, C., Priet, S., Ribet, D., Vernochet, C., Bruls, T., Lavialle, C. et al. (2008) A placenta-specific receptor for the fusogenic, endogenous retrovirus-derived, human syncytin-2. *Proc. Natl Acad. Sci. U.S.A.* **105**, 17532–17537 https://doi.org/10.1073/pnas.0807413105

10 Lokossou, A.G., Toudic, C. and Barbeau, B. (2014) Implication of human endogenous retrovirus envelope proteins in placental functions. *Viruses* **6**, 4609–4627 https://doi.org/10.3390/v6114609

11 Dupressoir, A., Marceau, G., Vernochet, C., Bénit, L., Kanellopoulos, C., Sapin, V. et al. (2005) Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae. *Proc. Natl Acad. Sci. U.S.A.* **102**, 725–730 https://doi.org/10.1073/pnas.0406509102

12 Heidmann, O., Vernochet, C., Dupressoir, A. and Heidmann, T. (2009) Identification of an endogenous retroviral envelope gene with fusogenic activity and placenta-specific expression in the rabbit: a new 'syncytin' in a third order of mammals. *Retrovirology* **6**, 107 https://doi.org/10.1186/1742-4690-6-107

13 Cornelis, G., Heidmann, O., Bernard-Stoecklin, S., Reynaud, K., Véron, G., Mulot, B. et al. (2012) Ancestral capture of *syncytin-Car1*, a fusogenic endogenous retroviral envelope gene involved in placentation and conserved in Carnivora. *Proc. Natl Acad. Sci. U.S.A.* **109**, E432–E441 https://doi.org/10.1073/pnas.1115346109

14 Dunlap, K.A., Palmarini, M., Varela, M., Burghardt, R.C., Hayashi, K., Farmer, J.L. et al. (2006) Endogenous retroviruses regulate periimplantation placental growth and differentiation. *Proc. Natl Acad. Sci. U.S.A.* **103**, 14390–14395 https://doi.org/10.1073/pnas.0603836103

15 Dunlap, K.A., Palmarini, M. and Spencer, T.E. (2006) Ovine endogenous betaretroviruses (enJSRVs) and placental morphogenesis. *Placenta* **27** (suppl.), 135–140 https://doi.org/10.1016/j.placenta.2005.12.009

16 Dunlap, K.A., Palmarini, M., Adelson, D.L. and Spencer, T.E. (2005) Sheep endogenous betaretroviruses (enJSRVs) and the hyaluronidase 2 (HYAL2) receptor in the ovine uterus and conceptus. *Biol. Reprod.* **73**, 271–279 https://doi.org/10.1095/biolreprod.105.039776

17 Armezzani, A., Arnaud, F., Caporale, M., di Meo, G., Iannuzzi, L., Murgia, C. et al. (2011) The signal peptide of a recently integrated endogenous sheep betaretrovirus envelope plays a major role in eluding gag-mediated late restriction. *J. Virol.* **85**, 7118–7128 https://doi.org/10.1128/JVI.00407-11

18 Murcia, P.R., Arnaud, F. and Palmarini, M. (2007) The transdominant endogenous retrovirus enJS56A1 associates with and blocks intracellular trafficking of Jaagsiekte sheep retrovirus Gag. *J. Virol.* **81**, 1762–1772 https://doi.org/10.1128/JVI.01859-06

19 Morozov, V.A., Morozov, A.V. and Lagaye, S. (2007) Endogenous JSRV-like proviruses in domestic cattle: analysis of sequences and transcripts. *Virology* **367**, 59–70 https://doi.org/10.1016/j.virol.2007.05.018

20 Baba, K., Nakaya, Y., Shojima, T., Muroi, Y., Kizaki, K., Hashizume, K. et al. (2011) Identification of novel endogenous betaretroviruses which are transcribed in the bovine placenta. *J. Virol.* **85**, 1237–1245 https://doi.org/10.1128/JVI.01234-10

21 Koshi, K., Suzuki, Y., Nakaya, Y., Imai, K., Hosoe, M., Takahashi, T. et al. (2012) Bovine trophoblastic cell differentiation and binucleation involves enhanced endogenous retrovirus element expression. *Reprod. Biol. Endocrinol.* **10**, 41 https://doi.org/10.1186/1477-7827-10-41

22 Cornelis, G., Heidmann, O., Degrelle, S.A., Vernochet, C., Lavialle, C., Letzelter, C. et al. (2013) Captured retroviral envelope syncytin gene associated with the unique placental structure of higher ruminants. *Proc. Natl Acad. Sci. U.S.A.* **110**, E828–E837 https://doi.org/10.1073/pnas.1215787110

23 Nakaya, Y., Koshi, K., Nakagawa, S., Hashizume, K. and Miyazawa, T. (2013) Fematrin-1 is involved in fetomaternal cell-to-cell fusion in Bovinae placenta and has contributed to diversity of ruminant placentation. *J. Virol.* **87**, 10563–10572 https://doi.org/10.1128/JVI.01398-13

24 Redelsperger, F., Cornelis, G., Vernochet, C., Tennant, B.C., Catzeflis, F., Mulot, B. et al. (2014) Capture of syncytin-Mar1, a fusogenic endogenous retroviral envelope gene involved in placentation in the Rodentia squirrel-related clade. *J. Virol.* **88**, 7915–7928 https://doi.org/10.1128/JVI.00141-14

25 Cornelis, G., Vernochet, C., Malicorne, S., Souquere, S., Tzika, A.C., Goodman, S.M. et al. (2014) Retroviral envelope syncytin capture in an ancestrally diverged mammalian clade for placentation in the primitive Afrotherian tenrecs. *Proc. Natl Acad. Sci. U.S.A.* **111**, E4332–E4341 https://doi.org/10.1073/pnas.1412268111

26 Cornelis, G., Vernochet, C., Carradec, Q., Souquere, S., Mulot, B., Catzeflis, F. et al. (2015) Retroviral envelope gene captures and *syncytin* exaptation for placentation in marsupials. *Proc. Natl Acad. Sci. U.S.A.* **112**, E487–E496 https://doi.org/10.1073/pnas.1417000112

27 Hayashi, K., Burghardt, R.C., Bazer, F.W. and Spencer, T.E. (2007) WNTs in the ovine uterus: potential regulation of periimplantation ovine conceptus development. *Endocrinology* **148**, 3496–3506 https://doi.org/10.1210/en.2007-0283

28 Sonderegger, S., Haslinger, P., Sabri, A., Leisser, C., Otten, J.V., Fiala, C. et al. (2010) Wingless (Wnt)-3A induces trophoblast migration and matrix metalloproteinase-2 secretion through canonical Wnt signaling and protein kinase B/AKT activation. *Endocrinology* **151**, 211–220 https://doi.org/10.1210/en.2009-0557

29 Lu, W., Tu, Z., Wang, S., Lu, J., Wang, Q., Wang, W. et al. (2013) Spatiotemporal expression of Wnt signaling pathway components during bovine placental development. *Theriogenology* **80**, 893–902 https://doi.org/10.1016/j.theriogenology.2013.07.015

30 Li, Q., Kannan, A., Das, A., Demayo, F.J., Hornsby, P.J., Young, S.L. et al. (2013) WNT4 acts downstream of BMP2 and functions via β-catenin signaling pathway to regulate human endometrial stromal cell differentiation. *Endocrinology* **154**, 446–457 https://doi.org/10.1210/en.2012-1585

31 Nayeem, S.B., Arfuso, F., Dharmarajan, A. and Keelan, J.A. (2016) Role of Wnt signalling in early pregnancy. *Reprod. Fertil. Dev.* **28**, 525–544 https://doi.org/10.1071/RD14079

32 Lu, J., Zhang, S., Nakano, H., Simmons, D.G., Wang, S., Kong, S. et al. (2013) A positive feedback loop involving Gcm1 and Fzd5 directs chorionic branching morphogenesis in the placenta. *PLoS Biol.* **11**, e1001536 https://doi.org/10.1371/journal.pbio.1001536

33 Matsuura, K., Jigami, T., Taniue, K., Morishita, Y., Adachi, S., Senda, T. et al. (2011) Identification of a link between Wnt/β-catenin signalling and the cell fusion pathway. *Nat. Commun.* **2**, 548 https://doi.org/10.1038/ncomms1551

34 Imakawa, K., Kusama, K. and Yasuda, J. (2014) Early placentation and local immune regulation. *Proceedings of the 9th Reproduction in Domestic Ruminants* (Juengel, J.L., Miyamoto, A., Price, C., Reynolds, L.P., Smith, M.F., Webb, R., eds), 25–29 August 2014, Obihiro, Japan. Context; 2014, 37K38

35 Bazer, F.W., Spencer, T.E. and Johnson, G.A. (2009) Interferons and uterine receptivity. *Semin. Reprod. Med.* **27**, 90–102 https://doi.org/10.1055/s-0028-1108013

36 Ideta, A., Urakawa, M., Aoyagi, Y. and Saeki, K. (2007) Early development *in utero* of bovine nuclear transfer embryos using early G1 and G0 phase cells. *Cloning Stem Cells* **9**, 571–580 https://doi.org/10.1089/clo.2007.0017

37 Nakagawa, S., Bai, H., Sakurai, T., Nakaya, Y., Konno, T., Miyazawa, T. et al. (2013) Dynamic evolution of endogenous retrovirus-derived genes expressed in bovine conceptuses during the period of placentation. *Genome Biol. Evol.* **5**, 296–306 https://doi.org/10.1093/gbe/evt007

38 Sakurai, T., Sakamoto, A., Muroi, Y., Bai, H., Nagaoka, K., Tamura, K. et al. (2009) Induction of endogenous interferon tau gene transcription by CDX2 and high acetylation in bovine nontrophoblast cells. *Biol. Reprod.* **80**, 1223–1231 https://doi.org/10.1095/biolreprod.108.073916

39 Ashton-Beaucage, D., Udell, C.M., Lavoie, H., Baril, C., Lefrançois, M., Chagnon, P. et al. (2010) The exon junction complex controls the splicing of MAPK and other long intron-containing transcripts in *Drosophila*. *Cell* **143**, 251–262 https://doi.org/10.1016/j.cell.2010.09.014

40 Sperber, G.O., Airola, T., Jern, P. and Blomberg, J. (2007) Automated recognition of retroviral sequences in genomic data—RetroTector©. *Nucleic Acids Res.* **35**, 4964–4976 https://doi.org/10.1093/nar/gkm515

41 Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. and Wold, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* **5**, 621–628 https://doi.org/10.1038/nmeth.1226

42 Kodama, Y., Shumway, M., Leinonen, R. and International Nucleotide Sequence Database Collaboration (2012) The sequence read archive: explosive growth of sequencing data. *Nucleic Acids Res.* **40**, D54–D56 https://doi.org/10.1093/nar/gkr854

43 Shimada, A., Nakano, H., Takahashi, T., Imai, K. and Hashizume, K. (2001) Isolation and characterization of a bovine blastocyst-derived trophoblastic cell line, BT-1: development of a culture system in the absence of feeder cell. *Placenta* **22**, 652–662 https://doi.org/10.1053/plac.2001.0702

44 Talbot, N.C., Caperna, T.J., Edwards, J.L., Garrett, W., Wells, K.D. and Ealy, A.D. (2000) Bovine blastocyst-derived trophectoderm and endoderm cell cultures: interferon tau and transferrin expression as respective *in vitro* markers. *Biol. Reprod.* **62**, 235–247 https://doi.org/10.1095/biolreprod62.2.235

45 Hambruch, N., Haeger, J.-D., Dilly, M. and Pfarrer, C. (2010) EGF stimulates proliferation in the bovine placental trophoblast cell line F3 via Ras and MAPK. *Placenta* **31**, 67–74 https://doi.org/10.1016/j.placenta.2009.10.011

46 Bai, H., Sakurai, T., Konno, T., Ideta, A., Aoyagi, Y., Godkin, J.D. et al. (2012) Expression of GATA1 in the ovine conceptus and endometrium during the peri-attachment period. *Mol. Reprod. Develop.* **79**, 64–73 https://doi.org/10.1002/mrd.21409

47 Nakagawa, S. and Takahashi, M.U. (2016) gEVE: a genome-based endogenous viral element database provides comprehensive viral protein-coding sequences in mammalian genomes. *Database* **2016**, baw087 https://doi.org/10.1093/database/baw087

48 Cohen, C.J., Lock, W.M. and Mager, D.L. (2009) Endogenous retroviral LTRs as promoters for human genes: a critical assessment. *Gene* **448**, 105–114 https://doi.org/10.1016/j.gene.2009.06.020

49 Sakurai, T., Bai, H., Bai, R., Arai, M., Iwazawa, M., Zhang, J. et al. (2012) Coculture system that mimics *in vivo* attachment processes in bovine trophoblast cells. *Biol. Reprod.* **87**, 1–11 https://doi.org/10.1095/biolreprod.112.100180

50 McLean, K.J., Crouse, M.S., Crosswhite, M.R., Black, D.N., Dahlen, C.R., Borowicz, P.P. et al. (2016) Expression of an endogenous retroviral element, during early gestation in beef heifers. *J. Anim. Sci.* **94**, 4452–4456 https://doi.org/10.2527/jas.2016-0793

51 Jerome-Majewska, L.A., Achkar, T., Luo, L., Lupu, F. and Lacy, E. (2010) The trafficking protein Tmed2/p24β$_1$ is required for morphogenesis of the mouse embryo and placenta. *Dev. Biol.* **341**, 154–166 https://doi.org/10.1016/j.ydbio.2010.02.019

52 Yamakoshi, S., Bai, R., Chaen, T., Ideta, A., Aoyagi, Y., Sakurai, T. et al. (2012) Expression of mesenchymal-related genes by the bovine trophectoderm following conceptus attachment to the endometrial epithelium. *Reproduction* **143**, 377–387 https://doi.org/10.1530/REP-11-0364

53 Kusama, K., Bai, R., Ideta, A., Aoyagi, Y., Okuda, K. and Imakawa, K. (2016) Regulation of epithelial to mesenchymal transition in bovine conceptuses through the interaction between follistatin and activin A. *Mol. Cell Endocrinol.* **434**, 81–92 https://doi.org/10.1016/j.mce.2016.06.017

54 Mangeney, M., Renard, M., Schlecht-Louf, G., Bouallaga, I., Heidmann, O., Letzelter, C. et al. (2007) Placental syncytins: genetic disjunction between the fusogenic and immunosuppressive activity of retroviral envelope proteins. *Proc. Natl Acad. Sci. U.S.A.* **104**, 20534–20539 https://doi.org/10.1073/pnas.0707873105

55 Ono, R., Kobayashi, S., Wagatsuma, H., Aisaka, K., Kohda, T., Kaneko-Ishino, T. et al. (2001) A retrotransposon-derived gene, PEG10, is a novel imprinted gene located on human chromosome 7q21. *Genomics* **73**, 232–237 https://doi.org/10.1006/geno.2001.6494

56 Youngson, N.A., Kocialkowski, S., Peel, N. and Ferguson-Smith, A.C. (2005) A small family of sushi-class retrotransposon-derived genes in mammals and their relation to genomic imprinting. *J. Mol. Evol.* **61**, 481–490 https://doi.org/10.1007/s00239-004-0332-0

57 Ono, R., Nakamura, K., Inoue, K., Naruse, M., Usami, T., Wakisaka-Saito, N. et al. (2006) Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. *Nat. Genet.* **38**, 101–106 https://doi.org/10.1038/ng1699

58 van der Kuyl, A.C., Mang, R., Dekker, J.T. and Goudsmit, J. (1997) Complete nucleotide sequence of simian endogenous type D retrovirus with intact genome organization: evidence for ancestry to simian retrovirus and baboon endogenous virus. *J. Virol.* **71**, 3666–3676 PMID:9094640

59 Lim, X., Tan, S.H., Koh, W.L.C., Chau, R.M.W., Yan, K.S., Kuo, C.J. et al. (2013) Interfollicular epidermal stem cells self-renew via autocrine Wnt signaling. *Science* **342**, 1226–1230 https://doi.org/10.1126/science.1239730

60 Matsui, T., Kinoshita-Ida, Y., Hayashi-Kisumi, F., Hata, M., Matsubara, K., Chiba, M. et al. (2006) Mouse homologue of skin-specific retroviral-like aspartic protease involved in wrinkle formation. *J. Biol. Chem.* **281**, 27512–27525 https://doi.org/10.1074/jbc.M603559200

61 Matsui, T., Miyamoto, K., Kubo, A., Kawasaki, H., Ebihara, T., Hata, K. et al. (2011) SASPase regulates stratum corneum hydration through profilaggrin-to-filaggrin processing. *EMBO. Mol. Med.* **3**, 320–333 https://doi.org/10.1002/emmm.201100140

62 Yang, J., Mowry, L.E., Nejak-Bowen, K.N., Okabe, H., Diegel, C.R., Lang, R.A. et al. (2014) β-Catenin signaling in murine liver zonation and regeneration: a Wnt-Wnt situation! *Hepatology* **60**, 964–976 https://doi.org/10.1002/hep.27082

63 Gregorieff, A., Pinto, D., Begthel, H., Destrée, O., Kielman, M. and Clevers, H. (2005) Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology* **129**, 626–638 https://doi.org/10.1016/j.gastro.2005.06.007