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Identification and characterization of an alternatively spliced variant of the MHC class I-related porcine neonatal Fc receptor for IgG

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Abstract

The neonatal Fc receptor for IgG (FcRn) functions to transport maternal IgG to the fetal/neonatal animals and protects IgG from catabolism. The present study identified two pFcRn cDNAs (1.071 kb and 0.795 kb) from intestinal epithelial cells. The corresponding mRNA transcripts were detected in porcine kidney cell line LLC-PK1, peripheral blood mononuclear cells and porcine tissues by RT-PCR and Northern blot. Sequence analysis showed that the 1.071 kb cDNA encodes the full-length pFcRn (pFcRn-L); whereas the 0.795 kb cDNA codes for a truncated pFcRn (pFcRn-S) with deletion of 92 amino acids matching to the alpha2 domain of pFcRn-L. The pFcRn-L was constitutively expressed by epithelial cells; however, pFcRn-S was not detectable in porcine tissues and cell lines although its transcript was abundant. Despite of the lack of native pFcRn-S, pFcRn-S was readily detected in transfected cells. Recombinant pFcRn-L was confirmed to bind IgG at pH 6.0, but not pH 7.5; however, pFcRn-S failed to bind IgG at both pH 5.0–6.0 and 7.5. The pFcRn-L was expressed on the cell surface and mainly localized in early endosomes. In contrast, pFcRn-S was absent from cell surface and primarily localized in the lysosome and pFcRn-S trafficking to lysosomes was independent of β 2m. The accumulation of pFcRn-S in the lysosome may explain the absence detection of native pFcRn-S protein expression. In addition, the trafficking of pFcRn-S to the lysosomal compartment suggests that in addition to sorting signals in its cytoplasmic tail, the FcRn structural integrity may be important for proper intracellular trafficking and function.

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³**Abbreviations used in this paper:** ER, endoplasmic reticulum; ECL, enhanced chemiluminescence; FcRn, neonatal Fc receptor; β 2m, β 2-microglobulin; IEC, intestinal epithelial cell; mAb, monoclonal antibody; IgG, immunoglobulin G; MHC, major histocompatibility complex; PDI, protein disulphide isomerase; PBS, phosphate buffer saline; RT-PCR, reverse transcription-PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Keywords

FcRn; epithelial; MHC; endosome; lysosome; porcine

1. Introduction

Maternal antibody plays a critical role in the protection of newborns from infectious diseases in the first few weeks or months of life, before the immune system becomes fully mature. Failure to passively transfer maternal antibodies can be devastating to the survival of newborns. Neonatal piglets are considered to be born agammaglobulinemic, although low levels of maternal IgG may pass the placenta into the fetus (1). Hence, newborn piglets acquire IgG from colostrum by absorbing through the intestine over a period of nearly 36 hour postpartum. Recent studies have also shown that the FcRn is expressed in the mammary gland and intestine of adult pigs (2, 3). The capability of the porcine FcRn to transport IgG across the intestine is verified by feeding piglets bovine IgG, which is detected in the pig circulation (3). FcRn, therefore, may play a major role in the passive acquisition of immunity in fetuses of some species and in newborns of most mammals [4–6]. In addition to its role in transporting IgG, FcRn functions to protect IgG and albumin from catabolism [5,7,8,9]. Although FcRn was initially reported in the intestinal epithelium of neonatal rodents, its expression has recently been identified in a variety of species, cell types and tissues, including epithelial cells, endothelial cells, macrophages and dendritic cells [10–14].

Similar to the major histocompatibility complex (MHC) class I and its related molecules, FcRn is composed of a heavy chain (HC) that is noncovalently attached to a light chain $\beta 2m$ (12 kDa) [15,16]. The overall exon-intron organization of the FcRn gene is similar to those of the MHC class I and its related molecules. The FcRn HC is composed of α_1 , α_2 , and α_3 external domains that are anchored to the cell surface by a short transmembrane domain and a cytoplasmic tail. Unlike MHC class I, FcRn is non-polymorphic and lacks a functional antigen peptide-binding groove. Instead, it is an Fc γ receptor exclusively for IgG. A significant feature of FcRn is that its interaction with IgG exhibits remarkable pH-dependence, i.e. binding IgG at acidic pH (6–6.5) and releasing IgG at neutral pH (7–7.4) [5,17,18].

Alternative splicing is a ubiquitous and essential mechanism for generating protein diversity and regulating protein expression. Alternative RNA splicing can occur in 3' or 5' untranslated regions, or in the protein coding sequence of a nascent mRNA. During RNA splicing, exons can either be retained in the mature message or targeted for removal in different combinations to create a diverse array of mRNAs from a single pre-mRNA. Insertion or deletion of the domains affects the protein-coding region of the mRNA [19]. Overall, alternative splicing of the primary mRNA allows the production of multiple, functionally-distinct proteins from a single gene. Splicing variants for MHC class I (HLA-A, -B, -C) and its related molecules (HLA-E, -F, -G, -Hfe, RT1-E, MR1, MIC-A, B, Zn-alpha 2-glycoprotein, CD1) have been reported [20–30].

In all cloned FcRn genes of all species thus far [5,10,11,13], most FcRn species have been reported to have a single mRNA species with a translated polypeptide of 45–150 kDa (45 kDa in humans and 50 kDa in rodents), except that there is a truncated bovine FcRn which lacks the transmembrane domain [13]. In the present study, we report that two mRNA species of porcine FcRn were expressed; the longer mRNA was the full-length transcript and the shorter mRNA represented an alternatively-spliced variant. However, we failed to detect the protein expression of this pFcRn splicing variant from porcine tissue and cell lines. Further analysis demonstrated that the absence of protein expression for pFcRn splicing variant might be resulted from the protein degradation in the lysosome.

2. Materials and Methods

2.1. Tissue specimens, cell lines, and antibodies

Peripheral blood mononuclear cells (PBMC) and tissue samples were collected from healthy pigs using the Animal Use Protocol approved by the Beltsville Area Institutional Animal Care and Use Committee, USDA (Beltsville, MD, USA). IPEC, a newborn piglet intestinal epithelial cell line, was a gift from Drs. Song Lu and Dennis D. Black [31], University of Tennessee Health Science Center, Memphis, TN, USA). LLC-PK1, a porcine kidney cell line, was a gift from Dr. Raktima Raychowdhury (Harvard Medical School, Boston, MA, USA). Cell lines were maintained in DMEM complete medium supplemented with 10 mM HEPES, 10% FCS (Sigma-Aldrich, MO, USA), 1% L-glutamine, nonessential amino acids, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. FO-1 cells (melanoma cell line) were from Dr. Richard S. Blumberg (Harvard Medical School, Boston, MA, USA) and grown in RPMI 1640 complete medium (Invitrogen, CA, USA) supplemented with 10 mM HEPES, 10% fetal bovine serum 1% L-glutamine, nonessential amino acids, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Rabbit anti-porcine FcRn serum antibody has been previously described [3]. Affinity-purified rabbit anti-FLAG epitope (DYKDDDDK, a single letter for amino acid) and mAb anti-FLAG M2 were purchased from Sigma-Aldrich (St. Louis, MO, USA); mouse anti-EEA1 from Transduction Laboratories (Lexington, KY, USA); mAb CDF4 (anti-Golgi 97) from Invitrogen; CyTM3-conjugated AffiniPure goat anti-mouse IgG and CyTM2-conjugated AffiniPure goat anti-rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); LysoTracker Red from Molecular Probes (Eugene, OR, USA). HRP-conjugated rabbit anti-mouse or donkey anti-rabbit antibody was from Pierce (Rockford, IL, USA). Mouse anti-PDI (protein disulphide isomerase) was a gift from Dr. Nicolas Bidere (National Institutes of Health, Bethesda, MD).

2.2 RT-PCR and Northern Blot

Cells were pelleted and resuspended at 10⁶ cells/ml in Tri-Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the method recommended by the manufacturer. The pFcRn gene was amplified by primers (Table 1, A) with a one-step RT-PCR kit (Qiagen, Valencia, CA, USA). PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide. Photographs were taken under UV light, using the Bio-Rad Gel/Chem Doc and software, Quantity One (Hercules, CA, USA). PCR-amplified products were excised and purified with the QIAquick® Gel Extraction Kit (Qiagen), cloned into pCDNA3 vector (Invitrogen), and sequenced.

For pFcRn mRNA detection, total RNA was isolated from fresh-isolated PBMC cells by Tri reagent. Poly (A) + RNA was prepared from total RNA (100 µg) using oligo d(T) magnetic beads (Invitrogen). Purified Poly(A)⁺ RNA was separated by electrophoresis on a 1.5% denaturing agarose gel and subjected to capillary transfer onto positively-charged nylon membranes. Transferred poly(A)⁺ RNA was then cross-linked to the membranes by UV irradiation and probed with a pFcRn cDNA fragment labeled with biotin (Ambion, Austin, TX, USA). Hybridization was performed overnight in ULTRA HybTM hybridization buffer (Ambion) at 42 °C. Unhybridized probe was removed by washing at up to 50 °C with 2X SSC (3M NaCl, 0.3M Sodium Citrate, pH 7.0), 1% SDS stringency washing buffer. Blots were incubated with streptavidin-HRP and visualized with ECL method by exposure to X-ray film (Kodak, Rochester, USA).

2.3. Preparation of cell lysates from porcine intestine and lung tissues

Cell lysates from porcine intestine and lung tissues were prepared by the method described previously [32,33]. In brief, the intestine and lung tissues were removed from the pig and rinsed with digestion buffer (Sigma) containing a cocktail of protease inhibitors (Roch) to detach epithelial cells. Brush borders were washed in PBS (pH 6.5) and resuspended in PBS (pH 6.5) containing 0.5% NP40, 10 mM iodoacetamide (Sigma), and a protease inhibitors. Cell debris was removed by centrifugation at 14,000 g at 4°C. The total protein concentrations were determined by the Bradford method (BioRad, Hercules, CA, USA) with BSA as a standard.

2.4. Construction of expression plasmids

The plasmid pCDNAFLAG was constructed by subcloning *Nhe-Xba* I fragments from pFLAGCMV (Sigma) into pCDNA3 (Invitrogen). This subcloning confers pFLAGCMV with neomycin-resistance. The pFcRn codons (23–356 amino acids, Figure 2A) were amplified by one-step RT-PCR from total RNA extracted from the IPEC cell line with primer pair C (Table 1). The upstream primer introduced a *Hind* III site and downstream primer an *Eco*R I site to facilitate cloning. Amplification was performed using one-step RT-PCR (Qiagen) with an initial incubation at 50°C for 30 min, then heating to 95°C for 15 min. The PCR was run by 35 cycles, each consisting of 95°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min, and terminated by a final extension step at 72°C for 10 min. The PCR product was purified by agarose gel electrophoresis using a gel extraction kit (Qiagen). The DNA fragment was digested with *Eco*R I and *Hind* III, and ligated into the plasmid pCDNAFLAG to generate the plasmid pCDNAFLAG-pFcRn-L or pCDNAFLAG-pFcRn-S. PCR primer pair E (Table 1) was used to construct a plasmid encoding FLAG-tagged FcRn mutant (amino acid 23–234) that lacks the cytoplasmic tail. The DNA fragment was digested with *Hind* III and *Eco*R I, and ligated into the plasmid pCDNAFLAG, to generate the plasmid pCDNAFLAGpFcRn-S-TD. In these plasmids, a preprotrypsin signal sequence and FLAG epitope were fused to the *N*-terminus of the pFcRn protein. The plasmid pEF6p β ₂M was constructed using porcine β ₂m primers (Table 1), in which the upstream primer introduced a *Bam*H I site and downstream primer a *Not* I site to facilitate cloning. The open reading frames of all plasmids were verified by sequence analyses.

2.5. Transfection and protein expression

Cell lines LLC-PK1 and FO-1 were transfected with pCDNAFLAGpFcRn-L, pCDNAFLAGpFcRn-S, pCDNAFLAGpFcRn-S-TD, or pEF6p β ₂M with Effectene transfection reagent (Qiagen), according to instructions from the manufacturer. Positive transfectants were tested for protein expression through Western blot, using anti-FLAG antibody. Stable transfectants were selected from single colonies in the presence of G418 and maintained in medium containing G418 at a concentration of 0.5–1 mg/ml. The stable cell line expressing pFcRn was designated as LLC-PK1-pFcRn-L or LLC-PK1-pFcRn-S.

2.6. Gel electrophoresis and Western blotting

Protein concentrations were determined by the Bradford method. The lysates were resolved using a 12% SDS-PAGE gel under reducing conditions, followed by transfer onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 5% non-fat milk, probed with affinity-purified FLAG Ab for 1 hr, followed by incubation with HRP-conjugated rabbit anti-mouse IgG or donkey anti-rabbit IgG. All blocking, incubation, and washing were performed in PBST solution (PBS and 0.05% Tween 20). Proteins were visualized by an ECL (Pierce) method, according to the instructions of the manufacturer.

2.7. IgG binding assay

IgG binding assays were performed as previously described (18) with the following modifications. Cells were lysed by shaking on ice for 1 hr in PBS (pH 5.0–6.0 or 7.5) containing 0.5% CHAPS (Sigma) and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Post-nuclear supernatants containing 1 mg of soluble proteins were incubated with pig IgG-Sepharose (Rockland Immunochemicals, PA, USA) at 4°C overnight. The unbound proteins were removed with PBS (pH 5.0–6.0 or 7.5) containing 0.1% CHAPS. The absorbed proteins were eluted with reducing sample buffer at 90°C for 5 min and were subjected to 12% SDS-PAGE analysis. Proteins were visualized by Western blotting using anti-FLAG Abs and ECL method (Pierce).

2.8. Cell surface biotinylation

Cell surface biotinylation was performed as previously described with modification [14]. LLC-PK1 cells (1×10^7) were suspended in 5 ml of PBS, pH 7.5, to which 2.5 ml sulfo-NHS-biotin in PBS (1 mg/ml) was added. The mixture was incubated at room temperature with rotation for 30 min. After washing with sodium phosphate buffer (pH 7.5), the cell pellet was resuspended in 1 ml of sodium phosphate buffer (pH 7.5) with 0.5% CHAPS. A post-nuclear supernatant was diluted 2-fold by sodium phosphate buffer (pH 7.5) with 0.1% CHAPS, then incubated with Avidin-Agarose (Pierce). Following washings, the bound protein was eluted with sample-loading buffer at 90°C and resolved by 12% reducing SDS-PAGE. A Western blot was performed with rabbit anti-FLAG Abs as described above.

2.9. Flow cytometry and confocal microscopy

Flow cytometry and confocal microscopy were performed as previously described [33]. For flow cytometry, pFcRn transfectants (5×10^5 cells/ml) were washed twice in suspension buffer (1% FBS in calcium-free PBS). The cell suspension was incubated with 10 μ g of anti-FLAG M2 mAb in a 500 μ l vol for 1 h, washed twice with suspension buffer, and stained with a fluorescein-labeled rabbit anti-mouse IgG. The cells were washed twice with suspension medium before fixation with 0.5% paraformaldehyde in PBS. Control staining with mock transfectants was performed for each analysis. Cells were analyzed using Beckman Coulter Cytomics FC500 and data were analyzed by FCS Express V3 software (Beckman Coulter, Fullerton, CA, USA).

For confocal microscopy, FO-1 cells and transfectants were cultivated on glass coverslips and maintained in serum-free medium for 48 h before intracellular staining. The coverslips were rinsed in PBS and cells were cold-fixed in 4% paraformaldehyde in PBS for 30 min at 4°C. For lysosome staining, cells were incubated with LysoTracker at recommended concentrations (50 $\times$ 75 nM) for 30 min prior to fixation. Subsequent procedures were done at room temperature. After two washings with PBS, the coverslips were immersed and permeabilized in solution (3% BSA and 0.2% Triton in PBS) for 30 min. Cells were incubated with affinity-purified rabbit anti-FLAG and anti-PDI, Golgi or EEA1 mAbs in PBST with 3% BSA for 1 h. Cells were then incubated with CyTM3-conjugated affiniPure goat anti-mouse IgG and CyTM2-conjugated AffiniPure goat anti-rabbit IgG in PBST with 3% BSA. After each step, cells were washed at least three times with 0.1% Tween-20 in PBS. To mount coverslips, the ProLongTM antifade kit was used (Molecular Probes, Eugene, OR, USA). Images were captured using a 100X oil-immersion objective on a Zeiss LSM 510 inverted microscope. The images were colorized and processed using the Zeiss LSM510 Imager Examiner software (Zeiss, Germany).

3. Results

3.1 Identification of long and short forms of pFcRn cDNA

The pFcRn was amplified from cDNA of total RNA isolated from cells including porcine intestinal IPEC (Fig. 1A, lane 2), kidney LLC-PK1 (lane 3), and peripheral blood mononuclear cells (PBMCs, lane 4), using primer pair A (Table 1) flanking the open reading frame (ORF) of pFcRn (GenBank accession number AY740682). Gel electrophoresis of the PCR products identified the presence of two cDNA fragments with distinctive sizes; the long fragment was the predicted full length 1071 bp cDNA encoding pFcRn ORF, the short fragment was possibly a truncated variant of the full-length version. To confirm this observation, Northern blot was performed using mRNA from PBMCs. Two distinctive transcripts (Fig. 1B) were also detected by Northern blot using $\alpha 1$ domain as a probe, apparently corresponding to the long and short forms of DNA detected by RT-PCR (Fig. 1A). To further verify that both of these mRNAs are indeed transcribed in vivo, we designed alternative PCR primer pair-B (Table 1) to amplify pFcRn cDNA fragments from porcine tissues. These freshly-prepared tissue RNA samples were derived from pig intestine (Fig. 1C, lane 1), lung (lane 2), spleen (lane 3), liver (lane 4), kidney (lane 5), muscle (lane 6), and heart (lane 7). As expected, two 558-bp and 282-bp DNA fragments were amplified (Fig. 1C). Negative control amplifications, using only a single primer or RNA without reverse transcription failed, to generate any products (Fig. 1D), suggesting the fidelity of PCR products. It was also noticed that the amount of pFcRn-S in PBMC was more abundant as detected by Northern blot than by RT-PCR method. The reason for this observation is not clear and needs further investigation using quantitative methods. Therefore, we concluded that pFcRn displayed two transcripts from both cell lines and tissues.

3.2 Splicing form of pFcRn lacks $\alpha 2$ domain

To sequence both cDNAs, we used primer pair A (Table 1) to amplify the entire open reading frame of pFcRn. Subsequent subcloning and sequencing of the long form of cDNA product (pFcRn-L) indicated it was identical to that of the full-length pFcRn cDNA, encoding a 356 amino acid polypeptide (GenBank accession number AY740682, [5]). The sequencing of the short form of cDNA product (pFcRn-S) showed pFcRn-S was indeed a truncated variant of the pFcRn-L, encoding a novel 264-amino acid polypeptide (Figure 2A, GenBank accession number EF208068). pFcRn-S has never been reported. The alignment of pFcRn-L and pFcRn-S amino acid sequences showed that both sequences were identical except that there was a deletion of 92 amino acids in the pFcRn-S. The deleted sequence in pFcRn-S corresponded to the $\alpha 2$ domain of pFcRn-L (Fig. 2B). Based on the differences in sequences, we also designed primer pair D (Table 1), in which the reverse primer is located in the $\alpha 2$ domain and the forward primer is positioned in $\alpha 1$ domain. RT-PCR with these primers would result in amplification of L- (458 bp), but not S-forms of pFcRn. Total RNAs extracted from different porcine tissues as indicated in Figure 1C were used. The results showed RT-PCR only amplified a single DNA product of 458 bp in the pig intestine (Fig. 2C, lane 1), lung (lane 2), spleen (lane 3), liver (lane 4), kidney (lane 5), muscle (lane 6), and heart (lane 7). Upon sequencing, it became evident that the amplified cDNA using primer pair D was identical to the predicted region (73–530 bp, GenBank accession number AY740682) of pFcRn-L cDNA. To further verify the pFcRn-S, the nucleotide sequence of the pFcRn-S (EF208068) was used to search the EST database using Megablast (optimized for highly similar sequences), (www.ncbi.nlm.nih.gov/blast). At least three EST sequences, BP142288.1, EW289930.1, and EW284029.1, were identified [34,35] and the nucleotides spanning $\alpha 2$ and $\alpha 3$ domain were perfectly aligned (Fig.2D). This suggests that the pFcRn-S lacks of $\alpha 2$ domain of the full length counterpart.

3.3. Expression of splicing form of pFcRn

To show whether pFcRn-S is expressed in porcine cell lines and tissues, cell lysates from LLC-PK1 cell line or porcine lung and intestinal tissues were subjected to Western blotting. As

shown in Figure 3, although rabbit anti-pFcRn serum antibody recognized full-length pFcRn-L protein from the lysates of lung or intestine tissue (A, *left panel*) and LLC-PK1 kidney cell line (A, *right panel*), we were unable to detect pFcRn-S protein in both tissues and cell line. This experiment was repeated by probed separately with affinity purified rabbit anti-FcRn peptide (CLEWKEPPSMRLKARP) Ab for 1 h, the same result were obtained. To further show whether pFcRn-S transcript can be translated into proteins, we subcloned both pFcRn-L and pFcRn-S cDNA fragments into the pcDNAFLAG vector (Fig. 3B). For detection of pFcRn protein, we engineered a FLAG epitope next to the N-terminus of pFcRn. Previous work showed that the fusion of FLAG epitope to the N-terminus of FcRn had no detectable effect on the function of human FcRn [18,33]. The pcDNAFLAG encoding pFcRn-S or -L was transfected into the porcine kidney cell line LLC-PK1. Protein bands of predicted sizes (40 and 29 kDa) were detected by FLAG-specific antibody in cell lysates by Western blot (Fig. 3C, lanes 1 and 2). When lysates of mock-transfected cells were subjected to Western blot analysis, no bands were detected (Fig. 3C, lane 3). Therefore, it was confirmed that the 40- and 29-kDa products represented pFcRn-L and pFcRn-S based on the molecular weights, respectively. This is consistent with nucleotide sequences of both pFcRn-L and pFcRn-S.

3.4. IgG-binding activity

FcRn binds IgG at acidic pH 6.0, but not at pH 7.5 [18]. Because pFcRn-S lacks $\alpha 2$ domain, we further examined whether pFcRn-S still retained its ability to bind IgG in a pH-dependent manner. IgG binding of pFcRn-S was assessed *in vitro* at either pH 5.0–6.0 or pH 7.5 in comparison with pFcRn-L, using different concentrations of cell lysates from the stable LLC-PK1-FcRn cell line. The detection of recombinant pFcRn proteins in the cell lysates confirmed the successful expression of pFcRn-L and pFcRn-S (Fig. 4, lanes 1 and 4). The IgG binding assay results showed that, as expected, the pFcRn-L strongly bound IgG at pH 6.0 (Fig. 4, lane 3), but not at pH 7.5 (lane 2). To eliminate the possibility that pFcRn-L might non-specifically bind to agarose beads, cell lysate from pFcRn-L transfectant was incubated with agarose beads without IgG. In this control, we failed to detect corresponding pFcRn protein bound to agarose beads under both pH conditions (data not shown), confirming the specificity of IgG binding. The pFcRn-S failed to bind IgG at both pH 5.0–6.0 and pH 7.5 (lanes 5 and 6) conditions. Anti-FLAG Abs against porcine pFcRn-L typically revealed two bands, as shown here, when pFcRn-L bound to IgG beads. The lower and upper bands may represent the high mannose oligosaccharide formed only in ER or high mannose, hybrid, and complex oligosaccharides formed in both ER and Golgi complexes, respectively. This is in agreement with other findings that two protein bands are detected in Western blot [5,33,36]. Only one band was detected in cell lysates of the pFcRn-S transfectant, which is consistent with the fact that the site for *N*-linked glycosylation in the $\alpha 2$ domain was absent in pFcRn-S.

3.5. Expression of pFcRn-L and pFcRn-S on cell surface

FcRn shows a predominant intracellular localization at steady state conditions, although some fraction can be detected on the cell surface [14]. Therefore, it is reasonable to determine if the deletion of $\alpha 2$ domain in pFcRn-S would result in alteration in its cellular localization. Expression of both FcRn-L and pFcRn-S was confirmed by Western blot (Fig. 5A, left panel, lanes 2 and 3). First, cell surface biotinylation experiments were performed and the results showed that pFcRn-S was not detectable on the cell surface of LLC-PK1-FcRn-S cells (Fig. 5A, right panel, lane 3). In contrast, pFcRn-L was detected at high levels on the cell surface of LLC-PK1-FcRn-L cells (right panel, lane 2). To confirm these results, the cell surface expression of pFcRn was further determined by flow cytometry. The cells were stained with either anti-FLAG mAb (Fig. 5B, dashed line) or an isotype control IgG1 (solid line). The results showed that high levels of pFcRn-L were detected on the cell surface of LLC-PK1-pFcRn-L (Fig. 5B, middle panel); in contrast, pFcRn-S was undetectable on the cell surface of LLC-PK1-pFcRn-S (Fig. 5B, right panel). These data suggest the cellular distribution of pFcRn-L

and pFcRn-S differed, at least in pig kidney cell line LLC-PK1. These results indicate that pFcRn-S was primarily expressed in the cytosolic compartment, possibly as a result of deletion of the $\alpha 2$ domain.

3.6. Subcellular localization of pFcRn-L and pFcRn-S

To further explore the differences in subcellular localization between pFcRn-L and pFcRn-S proteins, FO-1 cells were transiently co-transfected by pFcRn-L or pFcRn-S expression plasmid and porcine $\beta 2m$ expression plasmid. Subsequently, we co-localized pFcRn with organelle markers of the endoplasmic reticulum (ER, PDI), Golgi (GM97), the endosomal (EEA1) and lysosomal (LysoTracker) compartments. In the presence of $\beta 2m$ expression, pFcRn-L co-localized with early endosome (Fig. 6, merge of panel A+B), ER (merge of panel D+E), and Golgi (merge of panel G+H) markers. The pFcRn-L failed to co-localize with lysosome marker (Fig. 6, merge of panel J+K). The $\beta 2m$ also co-localized with ER and early endosome markers (data not shown). The pFcRn-L and $\beta 2m$ were found in vesicles that co-expressed EEA1, indicating that pFcRn-L must have exited the ER and trafficked to the early endosomal compartment. However, the majority of pFcRn-S was not well co-localized with the early endosome marker EEA1 (Fig. 6, merge of panel A+B); instead, pFcRn-S was co-localized with the Golgi (Fig. 6, merge of panel G+H) and lysosomal marker (Fig. 6, merge of panel J+K). Staining with an isotype-matched antibody showed no staining in the FO-1-pFcRn transfectant or the mock transfectant (data not shown). These observations indicate that pFcRn-L was mainly localized in the early endosomal compartment; whereas, pFcRn-S was primarily localized in the lysosomal compartment.

3.7. The trafficking of pFcRn-S is $\beta 2m$ independent

FcRn is a complex of heavy chain noncovalently associated with light chain $\beta 2m$, and the presence of $\beta 2m$ is critical for FcRn functions [6,16,33]. To determine whether pFcRn-S was associated with $\beta 2m$ molecule and that the association with $\beta 2m$ is important for pFcRn-S function, pFcRn-L and pFcRn-S were expressed in FO-1 cell line in the absence of $\beta 2m$ expression. The melanoma cell line FO-1 was used because it lacks $\beta 2m$ gene transcription and protein synthesis [37]. Similarly, we co-localized FcRn heavy chain with organelle markers of the ER, and the endosomal, Golgi, and lysosomal compartments. In the absence of $\beta 2m$ expression, pFcRn-L was primarily co-localized with the ER marker PDI (Fig. 7, merge of panel D+E), but not with the early endosome marker EEA1 (Fig. 7, merge of panel A+B) or lysosome tracker (merge of panel G+H). Without $\beta 2m$ expression, pFcRn-S was mainly co-localized with the Golgi and lysosomal markers (Fig. 7, merge of panel G+H). The trafficking pattern of pFcRn-S was very similar with $\beta 2m$ expression (Fig. 6). On the basis of these observations, we conclude that the co-expression of $\beta 2m$ was required for pFcRn-L to exit the ER and to be localized in the early endosome, but the co-expression of $\beta 2m$ was not important for trafficking of pFcRn-S.

3.7. The trafficking of pFcRn-S to the lysosomal compartment is dependent on its cytoplasmic tail

The cytoplasmic tail of pFcRn contains a typical dileucine-based motif [38]. This motif is typically exhibited in a number of endosomal/lysosomal resident proteins. It is possible that the cytoplasmic tail of the pFcRn-S molecule is also responsible for targeting FcRn-S to the lysosomal compartment. To examine this question, a truncated form, pFcRn-S-TD, lacking its cytoplasmic tail, was constructed and expressed in FO-1 cells (Fig. 8). Immunofluorescence studies indicated in the absence of cytoplasmic tail, pFcRn-S was mostly exhibiting as a honeycomb appearance, with little colocalization of early endosomal marker EEA1 (Fig. 8, *upper panel*) or lysosomal marker (Fig. 8, *middle panel*) in FO-1 cells. As shown in Figures 6 and Figure 7, the full-length of pFcRn-S was well colocalized with the lysosomal marker (Fig.

8, lower panel). Therefore, the cytoplasmic tail, perhaps the dileucine-based motif, of the pFcRn-S is most likely responsible for targeting the pFcRn-S protein to the lysosomal compartment.

4. Discussion

Alternative splicing, the procedure by which the exons of primary transcripts from genes can be spliced in different arrangements to generate structurally- and functionally-distinct mRNA and protein variants, may be one of the most broadly used mechanisms that explain the larger molecular complexity of higher eukaryotic organisms. Indeed, the recent bioinformatics analysis shows that 40–60% of transcribed genes possess alternate splice variants [19]. Thus, the biological significance of alternative spliced variants must be examined individually by characterization of the respective proteins. In this study, we characterized the biological properties of an alternative splicing variant for FcRn.

We demonstrated that porcine FcRn transcript gave rise to two distinct mRNAs. This was shown by several lines of evidence using complementary approaches. First, two DNA fragments spanning the entire pFcRn ORF, pFcRn-L and -S were amplified using a set of pFcRn-specific PCR primers from total RNA prepared from porcine epithelial cell lines (Fig. 1A), freshly-isolated PBMCs (Fig. 1A), and a variety of tissues (Fig. 1C). Although two transcripts were detected, we still cannot rule out the possibility that low levels of expression of the other splicing variants may also present. Second, it is possible that the pFcRn-S was generated by a low fidelity of RT-PCR reaction. However, identification of the two transcripts in PBMCs by Northern blot (Fig. 1B) clearly excluded this possibility. Third, the sequencing of several independent clones revealed that pFcRn-S was indeed a truncated form of full-length pFcRn. The alignment of amino acid sequences (Fig. 2B) showed that this truncated variant resulted from the pFcRn-L with deletion of 92 amino acids in the $\alpha 2$ domain. Fourth, the partial nucleotide sequences of pFcRn-S were aligned with at least three EST sequences (Fig. 2D). Therefore, pFcRn-S is likely the product of alternative mRNA splicing, possibly due to the skipping of exon encoding $\alpha 2$ domain during post-transcriptional modification. So far, among the FcRn homologues cloned from different species [5,10,11,13], only bovine FcRn mRNA had previously been shown to have a splicing variant and this truncated bovine FcRn lacked the transmembrane domain [13]. However, there is no further evidence that this bovine FcRn splicing transcript encoding a probable soluble product or it can be translated into a functional protein product. Although the pFcRn-S was fully expressed when its cDNA was transfected into porcine epithelial cells (Fig. 3), we were also unable to demonstrate whether the pFcRn-S protein was naturally expressed by porcine cell lines or tissues in our study. Incapable detection of pFcRn-S protein in its natural host might be caused by the quality and sensitivity of pFcRn antibody or rapid degradation of pFcRn-S protein in cells.

Intracellular trafficking of pFcRn-S in the transfectant is extremely intriguing. Newly-synthesized proteins in the ER are transported to the trans-Golgi network (TGN) and then targeted either directly to endosomes or to the cell surface. The only pathways to the lysosome are characterized by way of early endosomes and then late endosomes usually following endocytosis and by way of late endosomes direct from the TGN [39,40]. It is well known that specific motifs, such as tyrosine- or dileucine-based motifs, in the cytoplasmic domain of proteins play a critical role in the intracellular trafficking of membrane proteins. Two signals responsible for intracellular sorting have recently been recognized in the cytoplasmic domain of FcRn: one resembles a tyrosine-based motif, but with a tryptophan in place of the critical tyrosine residue; the other is a dileucine-based signal [36,41]. Recently, a Ca^{2+} -dependent calmodulin-binding motif in the cytoplasmic tail of FcRn was also reported (42). Amino acid-sequence alignment of the cytoplasmic tails of FcRn shows that both motifs are shared among FcRn species. Analysis of the amino acid sequence of the pFcRn-S revealed no apparent change

in membrane-spanning and the cytoplasmic domains (Fig. 2B). It may be, therefore, assumed that the splicing variant pFcRn-S would share a very similar intracellular transport behavior with pFcRn-L. Of greater interest, our findings showed that pFcRn-S was predominantly located in the lysosomal compartment; whereas, pFcRn-L was mainly detected in the early endosomal compartment (Fig. 6). The lysosomal location of pFcRn-S may help explain the failure to detect pFcRn-S in porcine tissues and cell line because of the possible degradation. It is possible that pFcRn-S might be sent to the cell surface, endocytosed, and sent via early endosomes to the lysosomes but with such low steady state levels at the surface and in early endosomes that it is not detected there, even by surface biotinylation. Decreased pFcRn-S in lysosomes following loss of the cytoplasmic endocytosis signals is consistent with this. It is also likely that the presence of pFcRn-S in lysosome was a result of its conformational change by lacking its $\alpha 2$ domain, or it might represent a misfolded version of the protein. Therefore, pFcRn-S may be missorted without routing to the early endosome. However, our results counteract the conjecture of the misfolding. First, the pFcRn-S contains a signal peptide which functions to channel pFcRn-S into the ER. If misfolded, pFcRn-S would be targeted to the protein degradation pathway because of an ER quality control mechanism. In general, misfolded ER proteins are retro-translocated from the ER to the cytoplasmic proteasome by an ubiquitin-dependent and Sec6-mediated pathway [43,44]. However, our data clearly showed that pFcRn-S resided in the lysosomal compartment; we also failed to detect any pFcRn-S on the cell surface (FIG. 5). Second, the dileucine-based motif has been considered to be an important signal for relocating proteins into endosomal/lysosomal compartments [40]. It is very likely that the dileucine-based motif in the cytoplasmic tail of pFcRn-S may direct pFcRn-S straight to the lysosome. In analyzing of a mutant pFcRn-S protein, the tailless pFcRn-S was failed to present in the lysosome (Fig. 8) in comparison with wild-type pFcRn-S, suggesting pFcRn-S protein was actively routing to the lysosome under the direction of its cytoplasmic tail. It remains a mystery as to why pFcRn-L routed to the endosomal, rather than lysosomal compartment, since both pFcRn-L and pFcRn-S proteins have identical intracellular cytoplasmic domains. We reason that, in addition to signal motifs in the cytoplasmic domain, the intact structure of FcRn, at least in pFcRn, may be one of the major determinants of the subcellular distribution of the FcRn. This conjecture is supported in our study. In the absence of $\beta 2m$ expression, pFcRn-L failed to exit to the ER compartment (Fig. 7). This is in agreement with our previous findings that the association with $\beta 2m$ is critical for exiting of FcRn from the ER and its appearance in the early endosome [33]. However, pFcRn-S protein was predominantly localized in the lysosomal compartment, regardless of $\beta 2m$ expression (Fig. 6 and Fig. 7). The observation that pFcRn-S exiting to the ER without $\beta 2m$ is interesting. It remains to know whether it dimerizes and gets out like MHC class II instead of MHC class I.

Several interesting issues may be perceived. First, many alternative splicing events occur in a specific tissue at a specific time in development and/or under certain physiological conditions. It is interesting to know the quantitative differences among tissues and cells in the expression of pFcRn-S. Second, FcRn has been shown to bind albumin at acidic pH and prolong its lifespan [9]. Because albumin is supposed to bind to a conserved His166 residue in the $\alpha 2$ domain (45), the pFcRn-S is not expected to bind albumin. Third, the expression of the splicing variants is highly regulated by inflammatory cytokines during inflammation, i.e., production of splicing variants of MHC class I molecules can be induced by proinflammatory cytokines [46,47]. Our recent data showed that FcRn expression can be regulated by cytokines such as TNF- α and IL-1 β [48]. Taking this into account, it would be interesting to know whether proinflammatory cytokines also regulate or change the patterns of FcRn splicing. Fourth, all MHC class I genes are organized similarly. Alternatively-spliced isoforms are known to exist for HLA-A and B, as well as HLA-G and the MHC class I-related gene, MR1, CD1 genes. The HLA-G gene encodes nine different variants [49]. Since similar domains are encoded by a single exon in the FcRn gene family, a detailed examination of the patterns and functions of splicing variants in other species is warranted to better understand the function and regulation of the FcRn system.

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Figure 1. Identification of long and short forms of pFcRn transcripts

(A). Detection of two isoforms of the porcine FcRn mRNA by RT-PCR amplification. Total RNA was extracted from porcine intestinal IPEC (lane 2), kidney LLC-PK1 (lane 3), and peripheral blood mononuclear cells (PBMCs, lane 4). FcRn cDNAs were amplified by one-step RT-PCR from 40 ng of total RNA with the indicated primer combinations. Primer pairs were designed to span the open reading frame of porcine FcRn gene. PCR conditions were described under "Experimental Procedures". Amplified products were separated on 2% agarose gels and stained with ethidium bromide. The data represents at least three independent experiments. The cDNA fragments corresponding to pFcRn-L and pFcRn-S are indicated by arrows.

(B). Detection of two pFcRn mRNA transcripts by Northern blot analysis. Poly(A)⁺ RNA was prepared from total PBMCs RNA (100 μ g) using oligo d(T) magnetic beads. The mRNA was separated on a formaldehyde agarose gel and probed with $\alpha 1$ domain cDNA labeled with biotin. The blot was then incubated with streptavidin-HRP and visualized with ECL method by exposure to X-ray film. The pFcRn-L and pFcRn-S are indicated by arrows.

(C). Detection of pFcRn cDNA in porcine tissues. pFcRn cDNAs were amplified by one step RT-PCR from 40 ng of total RNA extracted from the respective porcine tissues, including pig intestine (lane 1), lung (lane 2), spleen (lane 3), liver (lane 4), kidney (lane 5), muscle (lane 6), and heart (lane 7). Primer pairs were used to cover the $\alpha 1$, $\alpha 2$, and partial $\alpha 3$ open reading frame of pFcRn gene. The positions of the primers used are indicated by arrows in the schematic in the top panel. The pFcRn-L and pFcRn-S are indicated by arrows.

(D). RT-PCR detection of pFcRn splicing variants in intestine (lanes 1–4), lung (lanes 5–8), and spleen (lanes 9–12) total RNA. Reactions with a 5'-primer alone (lanes 4, 8, 12), 3'-primer alone (lanes 3, 7, 11) or without the reverse transcriptase (RT) enzyme (lanes 1, 5, 9) were included as negative controls. The cDNA fragments corresponding to pFcRn-L and pFcRn-S are indicated by arrows.

Figure 2. The splicing variant of porcine FcRn lacks α2 domain

(A). Nucleotide and deduced amino acid sequences of the short pFcRn-S transcript. Nucleotide sequence of the cDNA segments of pFcRn-L and pFcRn-S were amplified and sequenced. Sequence data of pFcRn-S have been deposited in the GenBank database under accession number EF208068. The nucleotide sequence of pFcRn-L obtained in the present study was identical to the full-length pFcRn sequence in GenBank (accession number AY740682) (data now shown).

(B). The alignment of porcine FcRn full-length (accession number AY740682) and splicing variant (accession number EF208068) amino acid sequences. Deleted amino acids are represented by dashes in the alternatively-spliced form of pFcRn. The amino acid sequence numbering starts with the first amino acid of the pFcRn genes. Numbering of amino acid residues is indicated on the *left*. The putative dileucine-based motif is underlined.

(C). RT-PCR using primer pair D (Table 1) covering the α1 and α2 open reading frame of pFcRn gene, resulting in amplification of the L-, but not S-forms of pFcRn. pFcRn cDNA was amplified by one-step RT-PCR from 40 ng of total RNA extracted from porcine tissues including pig intestine (lane 1), lung (lane 2), spleen (lane 3), liver (lane 4), kidney (lane 5), muscle (lane 6), and heart (lane 7). The positions of the primers used are indicated by *arrows* in the schematic in the top panel. The pFcRn cDNA is indicated by an *arrow*.

(D). The nucleotide sequences of the pFcRn-S were used in NCBI-BLAST searches of the GenBank EST database for the presence of splicing transcripts. At least three EST sequences, BP142288.1, EW289930.1, and EW284029.1, were identified. Nucleotides spanning α1 (in red) and α3 (in green) domain were aligned.

Figure 3. Expression of pFcRn-L and pFcRn-S proteins

(A). The detection of the porcine FcRn protein. Total cellular proteins (40 μ g) from lung (lane 1), intestinal epithelium (lane 2), and LLC-PK1 cell line (lane 3) were resolved on a 12% SDS-PAGE gel under reducing conditions. The proteins were transferred onto nitrocellulose membrane; blot was probed with rabbit anti-porcine FcRn antibody and visualized with ECL method. Molecular weight (kDa) is marked on the left margin. The similar experiments were performed at least three times.

(B). The cDNAs encoding porcine FcRn-L (23–356 amino acid) and FcRn-S (23–264 amino acid) were respectively amplified by RT-PCR primer pair C (Table 1) from the IPEC cell line, and the resultant DNA fragments were cloned into pcDNAFLAG expression plasmid. The cloned cDNA fragments were released from the vector by DNA restriction enzymes *Hind* III and *Eco*R I. The cDNA fragments corresponding to pFcRn-L and pFcRn-S are indicated by arrows, and the vectors are marked by an arrowhead.

(C). Expression of recombinant pFcRn-L and pFcRn-S proteins. LLC-PK1 cells were transfected with pcDNAFLAG (mock), pcDNAFLAG-pFcRn-L or pcDNAFLAG-pFcRn-S. The cell lysates (10 μ g) of pFcRn-S (lane 1), pFcRn-L (lane 2), and mock (lane 3) transfectants were subjected to SDS-PAGE gel electrophoresis under reduced conditions. Blots were probed with anti-FLAG antibody, and visualized with ECL method. The pFcRn-L and pFcRn-S proteins are indicated by arrows. The molecular weight (kDa) is marked on the left.

Figure 4. IgG binding assay

The lysates from pFcRn-L and pFcRn-S transfectants were made by mild detergent 0.5% CHAPS at pH 5–6 or pH 7, respectively. The 50 μ l of porcine IgG-conjugated beads were incubated with 0.5 ml lysates at a concentration of 0.5–1 mg/ml overnight at 4°C. After extensive washing, the beads were boiled with sample loading buffer. The samples were then subjected to SDS-PAGE gel electrophoresis under reduced conditions. The proteins were transferred onto nitrocellulose membrane, blotted with anti-FLAG antibody, and visualized with ECL method. The cell lysates from pFcRn-L (lane 1) and pFcRn-S (lane 3) transfectants or non-transfectant (lane 7) were used as controls, respectively. These experiments were performed at least three times.

Figure 5. Expression patterns of pFcRn-L and pFcRn-S on cell surface

These experiments were conducted in duplicate samples with identical results.

(A). Western blot analysis of pFcRn expression in surface biotinylated LLC-PK1 cells transfected with control plasmid (lane 1), pFcRn-L (lane 2), pFcRn-S (lane 3). Protein expressions were detected by Western blotting with Anti-FLAG antibody (left panel). Cell surface proteins were biotinylated (right panel) and solubilized by 0.5% CHAPS buffer. A post-nuclear supernatant was diluted and then incubated with avidin-agarose. Following washings, the bound protein was eluted with sample loading buffer at 90 °C and resolved by 12% SDS-PAGE under reducing conditions. A Western blot was performed with rabbit anti-FLAG Abs and developed with ECL.

(B). Flow cytometry analysis of pFcRn-L and pFcRn-S expression. LLC-PK1 transfectants were transfected with pCDNAFLAG encoding pFcRn-L or pFcRn-S and analyzed by flow cytometry. LLC-PK1 transfectants (5×10^5 cells/ml) were incubated with mAb anti-FLAG M2 antibody and with fluorescein-conjugated rabbit anti-mouse antibody. Cells were fixed with 0.5% paraformaldehyde in PBS. The dashed line represents staining of cells with anti-FLAG M2 mAb, and solid line represents cells stained with isotype-matched IgG. The staining was conducted twice with similar results.

Figure 6.

Subcellular Localization of porcine FcRn-L and pFcRn-S. FO-1 cells (5×10^5 cells/ml) were co-transfected with pCDNAFLAG encoding pFcRn-L or pFcRn-S in the presence of pEF6 β 2m. Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde and permeabilized in 0.2% Triton X-100. Subsequently, the cells were incubated with affinity-purified rabbit anti-FLAG (B, E, H and K, in green) specific antibody and mAb anti-EEA1 (A, in red) or PDI (D, in red), or GM97 (G, in red), followed by CyTM3-conjugated AffiniPure goat anti-mouse IgG and CyTM2-conjugated AffiniPure goat anti-rabbit IgG against IgG of the corresponding species. The lysosome is labeled after pulse-chase loading of lysosomes with LysoTracker (J, in red). Yellow signals are indicative of colocalization. These experiments were performed at least three times.

Figure 7.

Subcellular Localization of pFcRn-S in the absence of $\beta 2m$ expression. FO-1 cells (5×10^5 cells/ml) were transfected with pCDNAFLAG encoding pFcRn-L or pFcRn-S in the absence of pEF6 $\beta 2m$. Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde and permeabilized in 0.2% Triton X-100. Subsequently, the cells were incubated with affinity-purified rabbit anti-FLAG (B, E, H, and K, in green) specific antibody and mAb anti-EEA1 (A, in red), PDI (D, in red), or GM97 (G, in red), followed by CyTM3-conjugated AffiniPure goat anti-mouse IgG and CyTM2-conjugated AffiniPure goat anti-rabbit IgG against IgG of the corresponding species. The lysosome is labeled after pulse-chase loading of lysosomes with LysoTracker (J, in red). Micrographs of different focal planes are merged in C, F, I, and L. Yellow signals are indicative of colocalization. These experiments were performed at least three times.

Figure 8. The pFcRn-S presence in the lysosomal compartment is dependent on its cytoplasmic tail
 The plasmid encoding tail-less pFcRn-S-TD was constructed by PCR using pCDNAFLAG-pFcRn-S as a template as schematic representation in the top panel. FO-1 cells were transiently transfected with pCDNAFLAG encoding pFcRn-S-TD or pFcRn-S. Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde and permeabilized in 0.2% Triton X-100. Subsequently, the cells were incubated with affinity-purified rabbit anti-FLAG specific antibody and mAb anti- EEA1, followed by CyTM3-conjugated AffiniPure goat anti-mouse IgG. The lysosome was labeled after pulse-chase loading of lysosomes with Lyso Tracker. Yellow signals are indicative of colocalization. TM: transmembrane domain; CT: cytoplasmic domain; TD: tail deletion.

Table 1

PCR primers used in this study

Genes	Forward Primer	Reverse Primer	
pFcRn	5'-cccaagcttatgagggtccccggcct-3'	5'-cggaattctcatatcctaagattcagca-3'	(A)
	5'-cccaagcttgagacaaccacgcctccct-3'	5'-tcgggccttcacgcatgg-3'	(B)
	5'-cccaagcttgagacaaccacgcctccct-3'	5'-cggaattctcatatcctaagattcagca-3'	(C)
	5'-cccaagcttgagacaaccacgcctccct-3'	5'-aaggtcttctcctgttgac-3'	(D)
	5'-cccaagcttgagacaaccacgcctccct-3'	5'-cggaattcctatggcagcccttcctcatc-3'	(E)
β2m	5'-cgcgatccttcacaccgctccagtaacgatg-3'	5'-ataagaatcgggccgcttagtggtctcgaatccactaac-3'	