

Published in final edited form as:

*J Immunol.* 2012 May 1; 188(9): 4181–4187. doi:10.4049/jimmunol.1103509.

## IL-22 Is Expressed by the Invasive Trophoblast of the Equine (*Equus caballus*) Chorionic Girdle

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### Abstract

The invasive trophoblast cells of the equine placenta migrate into the endometrium to form endometrial cups, dense accumulations of trophoblast cells that produce equine chorionic gonadotropin between days 40 and 120 of normal pregnancy. The mechanisms by which the trophoblast cells invade the endometrium while evading maternal immune destruction are poorly defined. A gene expression microarray analysis performed on placental tissues obtained at day 34 of gestation revealed a >900-fold upregulation of mRNA encoding the cytokine IL-22 in chorionic girdle relative to noninvasive chorion. Quantitative RT-PCR assays were used to verify high expression of IL-22 in chorionic girdle. Additional quantitative RT-PCR analysis showed a striking increase in IL-22 mRNA expression in chorionic girdle from days 32 to 35 and an absence of IL-22 expression in other conceptus tissues. Bio-informatic analysis and cDNA sequencing confirmed the predicted length of horse IL-22, which carries a 39 extension absent in IL-22 genes of humans and mice, but present in the cow and pig. Our discovery of IL-22 in the chorionic girdle is a novel finding, as this cytokine has been previously reported in immune cells only. IL-22 has immunoregulatory functions, with primary action on epithelial cells. mRNA of IL-22R1 was detected in pregnant endometrium at levels similar to other equine epithelia. Based upon these findings, we hypothesize that IL-22 cytokine produced by the chorionic girdle binds IL-22R1 on endometrium, serving as a mechanism of fetal-maternal communication by modulating endometrial responses to trophoblast invasion.

The mechanisms that enable fetoplacental tissues to evade destruction by the maternal immune system are a longstanding focus of scientific investigation. In the decades since Medawar proposed the “fetus as allograft” model, reviewed by Billington (1), research has implicated a complex communication between trophoblast, maternal immune cells, and endometrium. Examples include production of IL-4 and IL-10 (2), HLA-G, IDO (3), and complement-regulatory proteins (4) by trophoblast, expression of RCAS1 by endometrium (3), and FOXP3<sup>+</sup> regulatory T cells at sites of trophoblast invasion (5).

Migration and endometrial invasion are attributes of trophoblast cells in many species, with the binucleate equine chorionic girdle (CG) cells being one example of this invasive phenotype (6). Deep invasion of trophoblast is thought to have played a role in human evolution by facilitating development of the human brain, yet this process also brings

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE35743.

### Disclosures

The authors have no financial conflicts of interest.

increased risk of immune-related placental dysfunction and diseases such as pre-eclampsia (7). Some immunomodulatory molecules (e.g., galectins) are proposed to have evolved in tandem with specific forms of placentation (7, 8). Trophoblast cells may also be novel sources of molecules produced by immune cells in adult organisms, as in the production of macrophage migration-inhibitory factor by human villous cyto-trophoblasts (9) and murine trophoblast giant cells (10).

Using gene expression array analysis comparing invasive and noninvasive equine trophoblast, we identified novel production of the immunomodulatory cytokine IL-22 by CG cells just prior to their migration through the endometrium to form the binucleate, chorionic gonadotropin-producing endometrial cups (11). IL-22 is a member of the IL-10 family of cytokines (12), and is involved in mucosal immunity and the maintenance and repair of epithelia (13–16). Since its first description in 2000 in human and mouse T cells (17, 18), IL-22 has been documented exclusively in immune cells, including Th subsets (Th17, Th22), NK cells, and bovine  $\gamma\delta$  T cells (13, 19–22).

IL-22 acts upon a heterodimeric receptor composed of its primary target IL-22R1 and IL-10R2 (23). This receptor is expressed on epithelial surfaces, including respiratory (24) and digestive tracts and skin (25). Binding of IL-22R1 by IL-22 activates transcription factors STAT3, STAT1, or STAT5 (26), and regulates genes associated with innate immunity (27) and cellular differentiation, migration, and survival (28, 29). A second receptor, IL-22R2, is a soluble binding protein thought to block downstream functions of IL-22 (30, 31). This study presents our initial microarray finding of IL-22 expression by CG cells, substantiates and expands upon this using quantitative RT-PCR (qRT-PCR) and bioinformatics, and identifies potential targets expressing IL-22R1 mRNA.

## Materials and Methods

### Animals

Mares of various breeds, ages, and parity were bred by artificial insemination to thoroughbred stallions using techniques previously described (32). All horses were owned by the Cornell Center for Equine Genetics and maintained in a herd setting. Procedures were performed in accordance with an animal care and use protocol approved by the Institutional Animal Care and Use Committee of Cornell University.

### Tissue preparation

Equine conceptuses were recovered by uterine lavage using a procedure previously described (33). Individual components, including CG, chorion, allantochorion, yolk sac, bilaminar omphalopleure, and fetus (Fig. 1), were isolated using a dissecting microscope. Endometrium was obtained with biopsy forceps following the conceptus recovery. Tissues were snap frozen in liquid nitrogen within one-half hour of collection. Epithelium and skin were obtained from animals of mixed age and breed euthanized in the Cornell University Hospital for Animals due to orthopedic conditions, but free of other diseases. Tissues were put on ice postmortem and snap frozen in liquid nitrogen within 1 h of collection. All samples were stored at  $-80^{\circ}\text{C}$  until processing. PBLs were isolated from heparinized jugular venous blood using a density centrifugation technique previously described (34). Stimulated lymphocytes were prepared in coculture with  $2.5\text{ }\mu\text{g/ml}$  PWM using a procedure described previously (35).

### RNA isolation and cDNA synthesis

RNA was isolated from samples using commercial kits (Qiashredder; RNeasy or RNeasy Plus Mini, Qiagen, Valencia, CA). All samples used in expression array experiments had

RNA integrity numbers >9.0, with most between 9.8 and 10.0, as determined by an Agilent Bioanalyzer (Agilent, Santa Clara, CA).

RNA samples isolated with the RNeasy kit were treated with DNase I (Invitrogen, Carlsbad, CA), whereas those isolated with RNeasy Plus were put over a genomic DNA removal column. First-strand cDNA synthesis was performed using M-MLV Reverse Transcriptase (USB, Cleveland, OH) following the manufacturer's protocol. All cDNA samples used in this study showed amplification of horse-specific  $\beta_2$  microglobulin and no evidence of genomic DNA contamination.

### Equine expression arrays

Two gene expression arrays developed on the Agilent (Agilent Technologies, Santa Clara, CA) platform were used, a commercial  $4 \times 44k$  array (Agilent array) and an  $8 \times 15k$  custom-designed product (Cornell array). For the Cornell array, horse sequences were obtained from three National Center for Biotechnology Information databases. Predicted reference sequences (RefSeqs) from the first assembly of the horse whole genome sequence (<http://www.broad.mit.edu/mammals/horse>) were combined with all horse UNIGENE entries, resulting in 24,628 sequences. All 37,316 available horse-expressed sequence tag sequences were combined with the RefSeq and UNIGENE entries to provide a transcriptome file for the eArray pipeline.

Horse-specific mRNA sequences were uploaded into the Agilent eArray custom microarray design pipeline (<https://earray.chem.agilent.com/earray/>), and duplicate sequences were filtered out. One probe was designed for each input sequence. Each probe received an Agilent quality score of 1 (highest) to 4 (lowest) and a cross-hybridization potential score of 0–1. A total of 14,307 probes with quality/cross-hybridization scores of 1/0, 1/1, or 2/0 was selected for the Cornell array.

### Array experiments

RNA was amplified linearly and Cy3 labeled using the Ambion Amino Allyl Message Amp II kit (Applied Biosystems, Foster City, CA), and then hybridized to both the Cornell and Agilent array slides using standard techniques. Data files were analyzed using GeneSpring GX11 software (Agilent). Samples were quantile normalized, and poorly performing probes were filtered out. The p values for fold changes were calculated using paired *t* tests. Results were exported into Excel (Microsoft, Redmond, WA) files.

### qRT-PCR assays

qRT-PCR assays used SYBR Green technology (Applied Biosystems) run on an Applied Biosystems 7500 Fast Real Time PCR instrument. Standard curves were generated using known copy numbers of a plasmid with a target gene-containing fragment. Primer sequences were designed with Primer3 (36) and are shown in Table I. SCAMP3 was used as a housekeeping gene.

### Cloning and sequencing of the equine IL-22-expressed gene sequence

IL-22 was amplified by RT-PCR from mitogen-stimulated equine lymphocytes. The PCR product was purified using QIAquick PCR Purification kit (28106) and cloned using the pcr4bluntTOPO vector transfected into Escherichia coli. Plasmid DNA was purified using QIAprep Spin Miniprep (27106), digested with EcoRI, and run on 1.5% agarose. Clones with bands at 570 bp were sequenced using the Sanger technique. Data were analyzed using Geneious Pro 5.0.4 (Biomatters) and Mega 5.05 (37–40).

## Results

### IL-22 is expressed by equine CG cells just prior to endometrial invasion

Global gene expression was compared between invasive (CG) and noninvasive (chorion) trophoblast (Fig. 1) from day 34 conceptuses. Four CG and chorion pairs were tested on the Cornell array, with three of the same pairs also tested on the Agilent array. Over 300 genes were differentially expressed between the two forms of horse trophoblast (D.C. Miller and D.F. Antczak, manuscript in preparation).

Our most striking result was high expression of IL-22 in CG relative to chorion, observed on all six probes used on both arrays. A single IL-22 probe on the Cornell array showed a 904-fold upregulation, whereas five IL-22 probes on the Agilent array had fold changes ranging from 665 to 1039 with a mean of 856 (Table II; array results deposited in Gene Expression Omnibus, reference GSE35743, <http://www.ncbi.nlm.nih.gov/geo/>). The novelty of our observation called for further exploration of IL-22 expression, the equine IL-22 gene, and the receptor components of the IL-22 system in the equine conceptus and endometrium.

To verify and expand upon our initial microarray finding, temporal expression of IL-22 was quantified in 15 CG-chorion pairs from days 32, 33, 34, and 35 of gestation using qRT-PCR. These points encompass the time when the CG is first grossly visible until the cells begin migrating into the endometrium. IL-22 mRNA was present in CG on day 32 and increased rapidly through day 35 (Fig. 2). Expression remained negligible in chorion and in trophectoderm from conceptuses recovered on days 15, 21, 25, and 30, prior to a grossly observable CG (data not shown). To evaluate spatial expression of IL-22, 3-d 34 equine conceptuses were dissected into their main component tissues, as follows: CG, chorion, allantochorion, yolk sac, bilaminar omphalopleure, and fetus (Fig. 1). Only the CG expressed high levels of IL-22 mRNA (Fig. 3).

IL-22 is produced by lymphocytes (23), prompting us to use stimulated horse lymphocytes as a proxy for comparison to assess physiologic relevance of the IL-22 levels in CG. Microarray analysis of lymphocyte mRNA produced hybridization signal intensities on the IL-22 probes comparable to those recorded for CG (Table II), and qRT-PCR analysis showed that peak IL-22 expression in a day 35 CG exceeded that of stimulated lymphocytes (Fig. 4).

To establish that CG samples were free from T lymphocyte contamination that could be the source of IL-22, the samples were evaluated for T lymphocyte marker CD3. CD3 was not detected in CG from days 32, 33, 34, or 35, although resting horse lymphocytes had 5153 copies of CD3 per 10 ng mRNA (data not shown).

### Horse IL-22 is similar to other species in gene structure and sequence

We explored whether the unusual expression of equine IL-22 in trophoblast might relate to peculiarities in its molecular structure or genomic environment. We confirmed the expressed coding sequence of equine IL-22 (Fig. 5) and found that alignment of the predicted protein with other species reveals a 3' extension of 10 aa relative to the human, mouse, and dog, but also observed in the cow and pig (Fig. 6).

The full equine IL-22 amino acid sequence showed greatest identity with the dog (*Canis familiaris*, 76%), followed by human (*Homo sapiens*, 75%), pig (*Sus scrofa*, 73%), mouse (*Mus musculus*, 69%), and cow (*Bos taurus*, 64%). Slightly higher sequence identities were observed if the 39 extension is excluded (Table III). A tree diagram further illustrates the relationships among the horse IL-22 protein sequence and the IL-22 sequences of these five

other species (Fig. 7). The predicted protein structure of the translated sequence is  $\alpha$ -helical as in other species (Fig. 8) (20, 23).

Equine IL-22 is located on equine chromosome 6 and contains five coding exons. The genomic landscape of IL-22 is similar to that of human and mouse, with IFNG, IL-26, and MDM1 in close proximity (Fig. 9) (41). In silico analysis of the promoter regions of the horse and several other species was performed. Species included in the analysis have varying forms of placentation, including human (discoid hemochorial), dog (zonary endotheliochorial), cow (cotyledonary synepitheliochorial), and pig (diffuse epitheliochorial, similar to the mare). The human IL-22 promoter sequence was obtained from the transcriptional regulatory element database (<http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home>). Transcriptional regulatory element database promoter sequence ID 9992 was searched against the horse (*Equus caballus*, TaxID 9796), dog (*C. familiaris*, TaxID 9615), cow (*B. taurus*, TaxID 9913), and pig (*S. scrofa*, TaxID 9823) genomes using the National Center for Biotechnology Information Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov>) discontinuous megaBLAST and default parameters. The sequence aligned only to regions overlapping the predicted IL-22 gene in all species (horse, E value 0.0, sequence identity 80%; dog, E value  $3e^{-51}$ , sequence identity 84%; cow, E value  $2e^{-160}$ , sequence identity 78%; pig, E value  $3e^{-36}$ , sequence identity 85%). Canonical regulatory elements conserved in all species included a TATA box (TATAWAWN) sequence. An initiator sequence (motif YYANWYY) was present with an identical sequence in the horse, cow, and pig. The same motif was present in the human with a single nucleotide difference, and it did not appear in the dog. CCAAT box motifs did not show conserved locations.

A computer-based transcription factor analysis of the promoter regions using AliBaba2 (<http://www.gene-regulation.com/pub/programs/alibaba2/intro.html>), TFSearch (<http://www.cbrc.jp/htbin/nph-tfsearch>), and SignalScan (<http://www.bimas.cit.nih.gov/molbio/index.shtml>) revealed STATX and AP-1 sites in all species examined (Fig. 10).

### IL-22R1 is expressed in endometrium at levels similar to other equine epithelial tissues

We investigated potential targets of CG IL-22 by assaying for receptor mRNA in adjacent tissues. qRT-PCR assays did not identify IL-22R1 in CG or other conceptus tissues, with the exception of chorion (Fig. 11A), as observed on the initial microarray (Table II). Assays were then run on endometrial tissue along with epithelium from the equine respiratory and gastrointestinal tracts and skin for comparison. IL-22R1 mRNA was detectable in days 32 and 34 pregnant endometrium at levels similar to epithelium (Fig. 11B), where it is expected to be found (25, 42).

qRT-PCR analysis of conceptus tissues for IL-22R2 showed minimal expression in fetal tissues (data not shown). CG and chorion showed no expression of this molecule, with 2803 copies per 10 ng mRNA in stimulated equine lymphocytes, consistent with our original microarray finding (Table II).

## Discussion

To our knowledge, our discovery that IL-22 is expressed by the CG cells of the equine trophoblast is the first clear evidence for production of this cytokine by a nonimmune cell type. IL-22 was first identified in lymphocytes of the mouse and the human (17, 18) and subsequently in other lymphoid populations (20, 21, 23, 43–46). Attempts to identify IL-22 in nonhematopoietic cells were unsuccessful (23). Taken together, to our knowledge, our data provide the first clear evidence for expression of IL-22 by a non-immune cell type. We show consistently high expression of IL-22 in CG using both gene expression arrays and qRT-PCR assays. Rapid upregulation of IL-22 just prior to endometrial invasion and



restriction of expression to CG suggests a specific physiologic function for IL-22 at this stage of placental development. An IL-22 mRNA level in CG comparable to that found in stimulated lymphocytes supports the physiologic relevance of our finding.

Expression of IL-22 in CG is a novel example of trophoblast co-opting an existing immunoregulatory gene for a physiologic need specific to placentation. A role for IL-22 has been implicated in human reproduction, but in these instances is produced by uterine NK cells (47). Our finding may represent a mechanism specific to endometrial cup biology in equids or more broadly to epitheliochorial placentation. It is clear from the pattern of expression we observed that IL-22 mRNA is detectable for a brief interval, and possibly the appropriate samples have not been evaluated in other species.

Although the structure and coding sequence of equine IL-22 are similar to other species, a notable characteristic of the predicted equine IL-22 protein is its extra 10 aa relative to the human, mouse, and dog. The longer amino acid sequence was considered unique in cows by researchers investigating bovine IL-22 (20). We demonstrate that this extension is also present in the horse and pig. It is of note that the three species having the 3' extension do not share the greatest sequence identity of the IL-22 protein (refer to Table III, Fig. 7). This raises questions as to the conservation of function associated with this extension. These three species do, however, share identical regulatory motifs in their upstream regions. In some reproductive hormones, modifications to C-terminal extensions affect receptor affinity (48). Additional investigation is necessary to determine whether this modification has functional significance in ungulate IL-22.

Current understanding of IL-22 regulation is based largely upon Th17 and other immune cell pathways, known to involve STATX, AP-1, ROR $\gamma$ t, IL-6, IL-23, AhR, and other molecules (20, 45, 49). Of these, only AhR showed significant expression in CG on our original array. STATX and AP-1 motifs were identified in the promoter regions of the horse and other species examined in our in silico analysis. Further research must be undertaken to define the mechanisms upstream and downstream of IL-22 expression in the CG.

Binding of IL-22R1 activates STAT pathways that regulate proteins involved in antimicrobial defense, cell differentiation, and cell migration (46). In vitro studies using reconstituted human epithelium indicate a role for IL-22 in the healing of epithelial surfaces (29). The overlap of physiologic processes involved in CG cell formation of the endometrial cups and IL-22 functions in other cell types allows plausible hypotheses for IL-22 function in the CG cells. From early specialized CG cell development on day 25 to final formation of the endometrial cups by day 42, CG cells undergo extensive differentiation and migration. Mononuclear cells terminally differentiate into binucleate, equine chorionic gonadotropin-producing cells of the functional endometrial cup and migrate through the endometrial epithelium and into the stroma (11, 50, 51). The disrupted endometrium then re-epithelializes (51, 52). The presence of IL-22 in the CG and IL-22R1 in the maternal endometrium lends itself to the hypothesis that IL-22 facilitates re-epithelialization of the endometrium after trophoblast migration. IL-22 also may be involved in the uterine innate immune system. IL-22 upregulates antimicrobial proteins such as  $\beta$ -defensins at mucosal surfaces (25, 46). Maternal-fetal cooperation in immune response to infection has been documented previously (53, 54). Localized disruption of endometrium at the site of trophoblast invasion could be a route for infection, counteracted by stimulation of antimicrobial molecules. Finally, the presence of IL-22R1 in chorion could indicate a role for IL-22 in the development of the girdle itself.

IL-22R2 has been identified in various tissues, including human placenta and lymphatic tissues. It has multiple splice variants in other species, and in vivo functions that remain undefined (42). A role for IL-22R2 in equine placentation is not immediately evident.

In summary, our data establish dramatic IL-22 expression by a nonimmune cell type, the invasive trophoblast of the equine CG. We hypothesize that IL-22 from the chorionic girdle cells facilitates equine placental development by initiating repair of the endo-metrial epithelium at the sites of trophoblast invasion.

## Acknowledgments

We thank breeding technician Emily Silvela and Dr. Leela Noronha for the CD3 assay.

This work was supported by National Institutes of Health Grants R01-HD049545, T32-RR007059, and K08-HD065914 and the Zweig Memorial Fund. D.F.A. is an investigator of the Dorothy Russell Havemeyer Foundation.

## Abbreviations used in this article

CG	chorionic girdle
qRT-PCR	quantitative RT-PCR

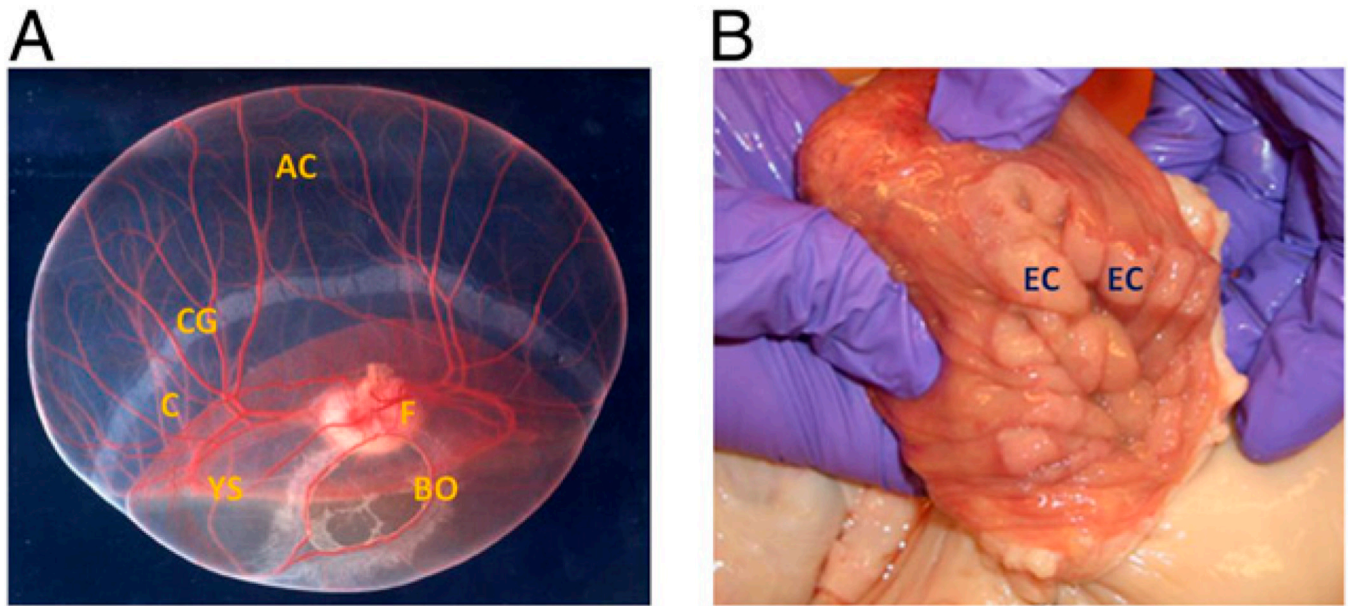
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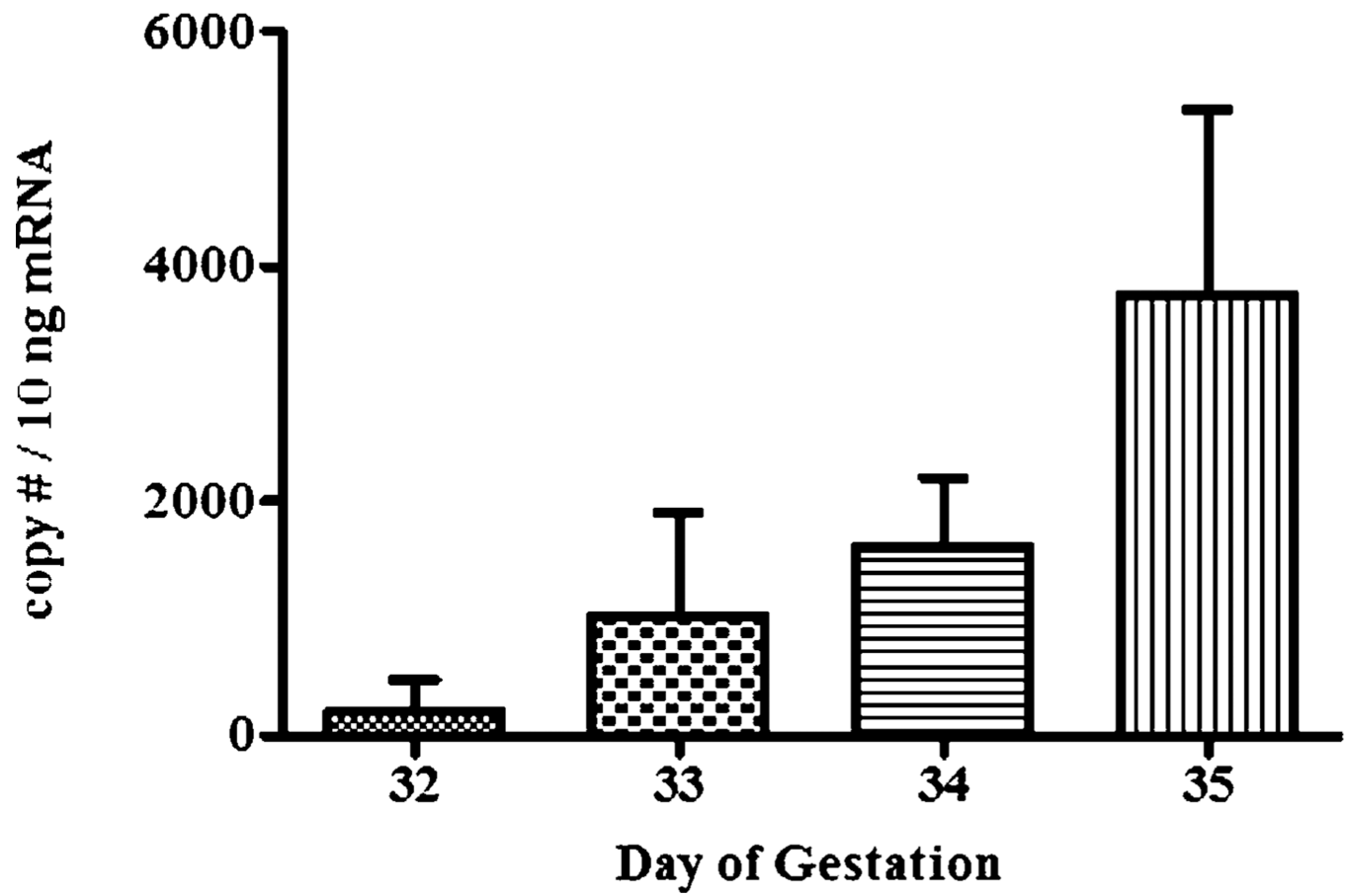


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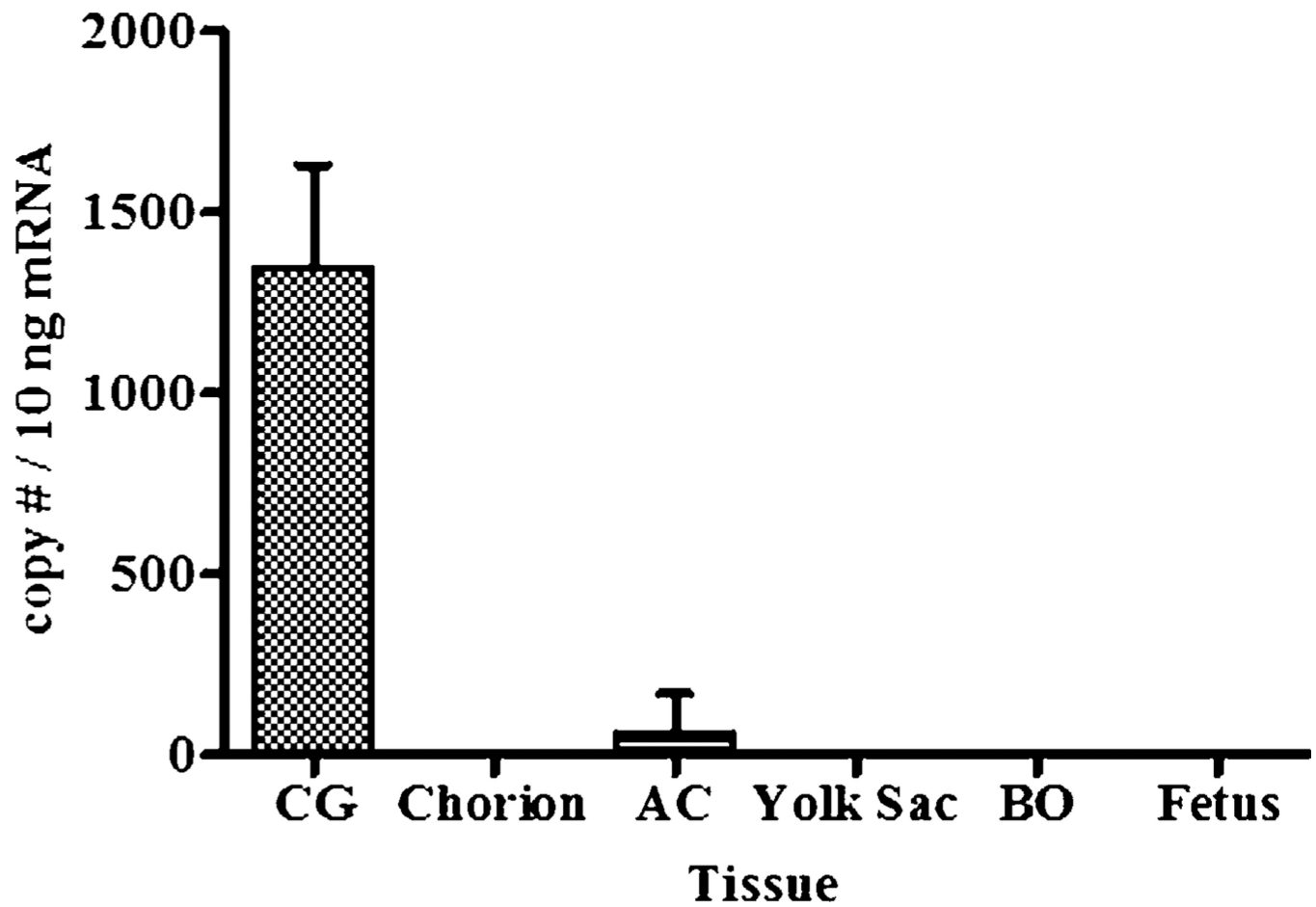
**FIGURE 1.**

Gross specimens of horse conceptus and endometrial cups in situ. (A) Day 34 horse conceptus showing CG as a distinct pale band encircling the conceptus, chorion (C), allantochorion (AC), yolk sac (YS), bilaminar omphalopleure (BO), and fetus (F). The CG cells begin migrating from the conceptus through the endometrium at days 36–38 to form (B) the equine chorionic gonadotropin-producing endometrial cups (EC), shown here within the maternal endometrium at day 44 of gestation in a postmortem specimen.



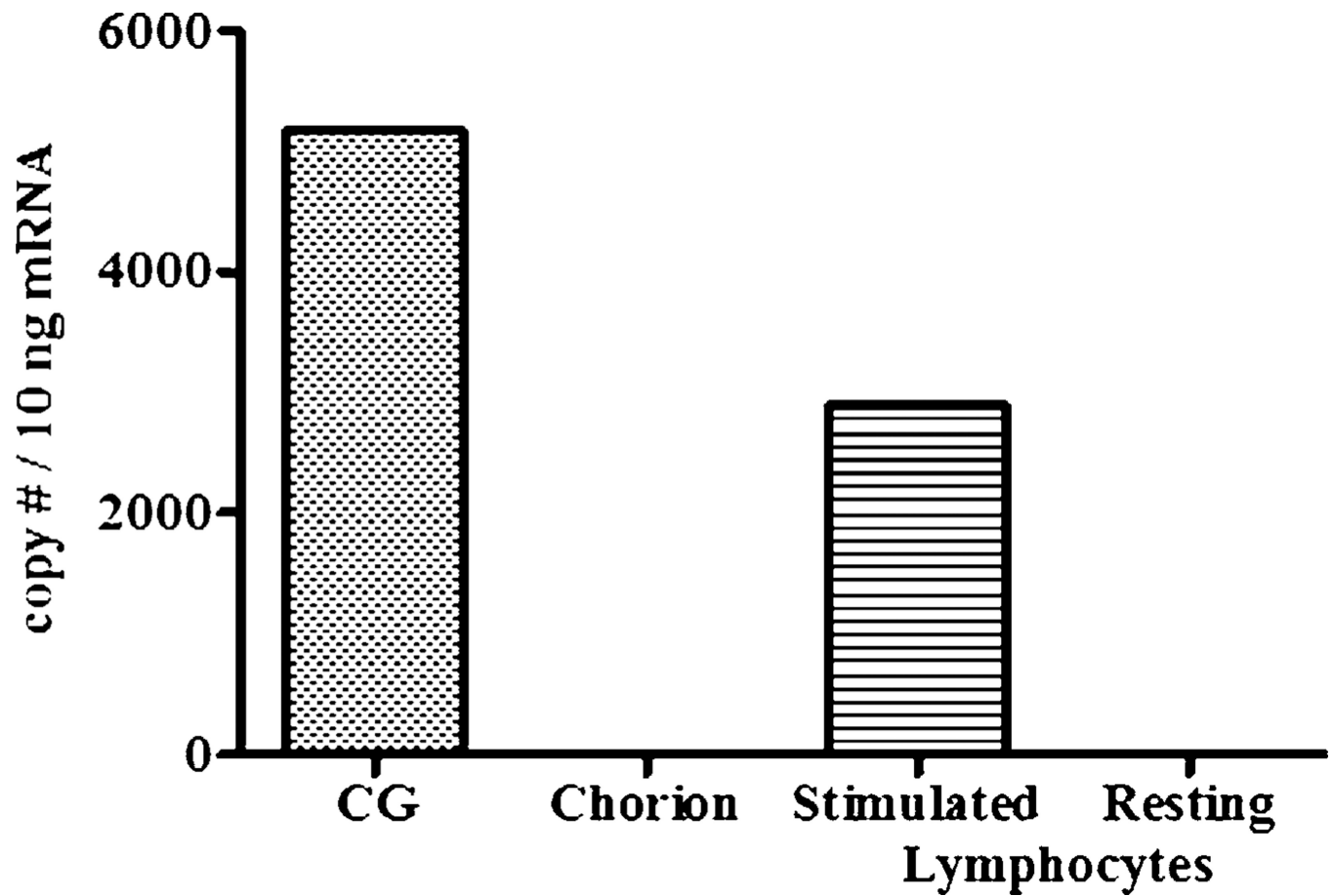
**FIGURE 2.**

Temporal analysis of IL-22 expression in the CG. CG samples from days 32 ( $n = 3$ ), 33 ( $n = 3$ ), 34 ( $n = 5$ ), and 35 ( $n = 4$ ) show IL-22 expression increasing dramatically just prior to invasion of the endometrium. Copy numbers represent means of two separate quantitative PCR experiments run in triplicate for each sample. Chorion membrane from each conceptus also was tested (data not shown, mean = 3.7 copies/10 ng mRNA, range = 0–30 copies/10 ng mRNA). Trophoblast from days 15, 21, 25, and 30 taken from the approximate area where the CG arises also showed negligible expression of IL-22 (data not shown,  $n = 6$ , mean = 3.7 copies/10 ng mRNA, range = 0–10 copies/10 ng mRNA).



**FIGURE 3.**

Spatial analysis of IL-22 expression in the day 34 horse conceptus. Additional tissues from three day 34 conceptuses were analyzed to assess the spatial pattern of IL-22 mRNA expression, including allantochorion (AC), yolk sac, bilaminar omphalopleure (BO), and fetus. The high expression of IL-22 in the horse conceptus is restricted to the CG. Samples were run in triplicate.



**FIGURE 4.**

IL-22 expression in day 35 CG and chorion and horse lymphocytes. Quantitative PCR assays demonstrate that peak production of IL-22 in the CG exceeds that of stimulated equine lymphocytes. Data represent individual samples run in triplicate.



*Exon 1* -----  
 A T G G C C A C C C T G C A G A A A T T T T T G A G C T C T T G C C T C C T G G G G A C T C T G G C [ 50]  
 -----  
 A G C C A G C T G C C T C C T T C T C G T T G C C C T G T G G G T G C A G G G A G G A G T G G C T G [ 100]  
 -----  
 C G C C C C T T G G C T C T C A C T G C A G G C T T G A C G A G T C C G A C T T C C A G C A G C C A [ 150]  
 -----  
 T A C A T C A T C A A C C G C A C C T T C A A G C T G G C C G A C G A G G C T A G T T T G G C A G A [ 200]  
 -----  
 T A A C A A C A C A G A T G T T C G G C T C A T T G G G G A G A A A C T G T T C C A T G G A G T C A [ 250]  
 -----  
 A T G T G A G A C A G C A C T G C T A C C T G A T G A A G C A G G T G C T A A A C T T T A C C C T T [ 300]  
 -----  
 G A A G A G G T G C T G C T C C C T T A C T C T G A C C G A T T C C A G C C T T A C A T G C A G G A [ 350]  
 -----  
 G G T G G T G T C C T T C C T G G C A G G G C T C A G C A A C A A G C T A A G C C A A T G C C A T A [ 400]  
 -----  
 T T G A G G G T G A T G A C C A G C A T G T C C A G A A A A A G T G C A A A A C C T G A A G G A C [ 450]  
 -----  
 A C A G T G A A A A A G C T T G G A G A G A G T G G A G A C A T C A A A G C A A T T G G G G A A C T [ 500]  
 -----  
 G A A T T T G C T G T T T A T G C G G C T G A A A A A T G C C T G C A T T G G A C C A G A G A A A [ 550]  
 -----  
G C T G G A A A A T G G A T A A C T A A [ 570]

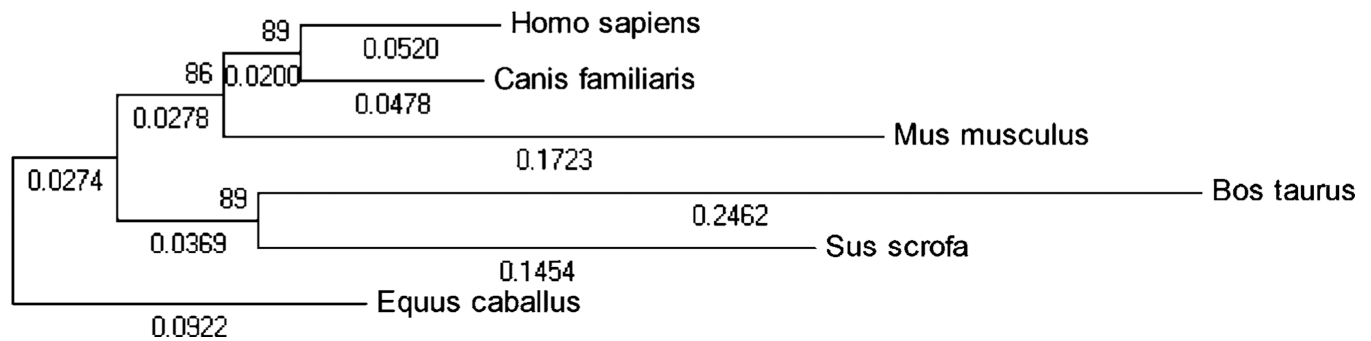
**FIGURE 5.**

Nucleotide sequence of horse IL-22. The coding sequence of horse IL-22 is shown as determined from mRNA isolation and cDNA synthesis. Five coding exons are labeled, and nucleotides producing the 10-aa 3' extension peculiar to ungulate IL-22 are underlined.

Equus caballus	M	A	T	L	Q	K	F	L	S	S	C	L	L	G	T	L	A	A	S	C	L	L	L	V	A	L	W	V	Q	G	G	V	A	A	P	L	G	S	H	C	R	L	D	E	S	D	F	Q	Q	P	[ 50]	
Bos taurus	.	.	A	.	.	S	V	G	.	P	.	R	D	.	.	.	G	.	.	.	.	.	.	V	M	V	.	C	A	.	R	.	A	.	.	.	I	T	.	.	.	N	.	.	.	E	.	[ 50]				
Sus scrofa	.	.	A	.	R	T	S	G	.	P	F	.	E	.	.	.	.	.	.	.	.	.	.	L	.	A	.	.	.	V	.	I	T	H	.	.	K	.	Q	.	N	.	.	.	[ 50]							
Homo sapiens	.	.	A	.	.	S	V	.	F	.	M	.	.	.	.	T	.	.	.	.	.	.	.	L	.	L	.	.	A	.	.	.	I	S	.	.	.	.	K	.	N	.	.	[ 50]								
Mus musculus	.	.	V	.	.	S	M	.	F	S	.	M	.	.	.	.	.	.	.	.	.	.	.	I	.	.	A	.	E	A	N	.	L	.	V	N	T	R	.	K	.	E	V	.	N	.	[ 50]					
Canis familiaris	.	.	A	.	.	S	V	.	.	T	.	M	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	A	.	L	.	I	S	.	.	.	.	.	K	.	N	.	.	[ 50]							
Equus caballus	Y	I	.	I	N	R	T	F	K	L	A	D	E	A	S	L	A	D	N	N	T	D	V	R	L	I	G	E	K	L	F	H	G	V	N	-	V	R	Q	H	C	Y	L	M	K	Q	V	L	N	F	T	[100]
Bos taurus	.	.	F	.	H	.	.	T	.	Q	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	I	H	Q	.	T	K	R	.	V	L	.	.	.	.	I	[100]					
Sus scrofa	.	.	T	.	.	.	.	T	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	N	.	Q	.	.	.	-	M	.	E	R	.	.	V	.	.	.	[100]						
Homo sapiens	.	.	T	.	.	.	.	M	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	-	M	S	E	R	.	.	.	.	.	.	.	[100]							
Mus musculus	.	.	V	.	.	.	.	M	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	S	-	A	K	D	Q	.	.	.	.	.	.	[100]								
Canis familiaris	.	.	T	.	.	.	.	M	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	M	G	E	R	.	.	.	E	.	.	.	[100]								
Equus caballus	L	E	E	V	L	L	P	Y	S	D	R	F	Q	P	Y	M	Q	E	V	V	S	F	L	A	G	L	S	N	K	L	S	Q	C	H	I	E	G	D	D	Q	H	V	Q	K	K	V	Q	N	L	K	[150]	
Bos taurus	.	.	.	.	.	F	.	Q	.	K	.	H	.	.	E	K	.	P	.	F	S	R	.	K	.	.	.	.	.	V	.	S	.	N	.	.	I	.	R	N	.	.	.	[150]								
Sus scrofa	.	.	.	.	.	F	.	N	.	.	H	.	.	.	.	A	.	.	D	S	.	K	.	.	.	.	.	R	.	K	.	.	.	.	I	.	R	N	.	N	.	F	.	[150]								
Homo sapiens	.	.	.	.	.	F	.	Q	.	.	.	.	.	.	.	P	.	.	R	.	R	.	T	.	.	.	.	.	.	.	.	L	.	I	.	R	N	.	K	.	[150]											
Mus musculus	.	.	D	.	.	.	.	Q	.	.	.	.	.	.	.	P	.	T	K	.	Q	.	S	.	.	.	.	S	.	.	.	N	I	.	.	N	.	R	R	.	[150]											
Canis familiaris	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	P	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	I	.	R	N	.	K	.	[150]									
Equus caballus	D	T	V	K	K	L	G	E	S	G	D	I	K	A	I	G	E	L	N	L	L	F	M	R	L	K	N	A	C	I	G	P	E	K	S	W	K	M	D	N	[190]											
Bos taurus	N	.	.	.	.	.	.	E	.	.	V	.	.	.	.	.	.	.	.	.	.	T	T	.	R	E	.	A	Q	V	D	Q	G	.	.	G	Y	[190]														
Sus scrofa	.	.	.	.	.	.	.	E	.	.	V	.	.	.	Y	.	.	.	.	.	.	A	.	.	E	.	T	L	.	G	H	.	.	.	.	.	[190]															
Homo sapiens	.	.	.	.	.	.	.	E	.	.	.	.	.	.	D	.	.	.	.	.	.	S	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	[190]														
Mus musculus	E	.	.	.	.	.	.	E	.	.	.	.	.	.	D	.	.	.	.	.	.	S	.	R	.	.	V	.	.	.	.	.	.	.	.	.	.	[190]														
Canis familiaris	.	.	Q	.	.	.	N	E	.	.	.	.	.	.	D	.	.	.	.	.	.	A	.	R	.	.	V	.	.	.	.	.	.	.	.	.	.	[190]														

**FIGURE 6.**

Comparative alignment (ClustalW) of IL-22 from the domestic horse and other species. Horse IL-22 shows a 3' extension also observed in bovine and predicted porcine IL-22, but not in other species. Consensus is indicated by a dot, and a dash indicates a gap. The sequence within the box represents the non-IFN IL-10 family signature. (*E. caballus* RefSeq, XP\_001491804; *B. taurus* GenBank, DAA29799; *S. scrofa* RefSeq, XP\_001926191.1; *H. sapiens* GenBank, AAQ8924.1; *M. musculus* GenBank, AAI16236.1; *Canis lupus familiaris* RefSeq, XP\_538274.1.) Analysis performed with MEGA5.

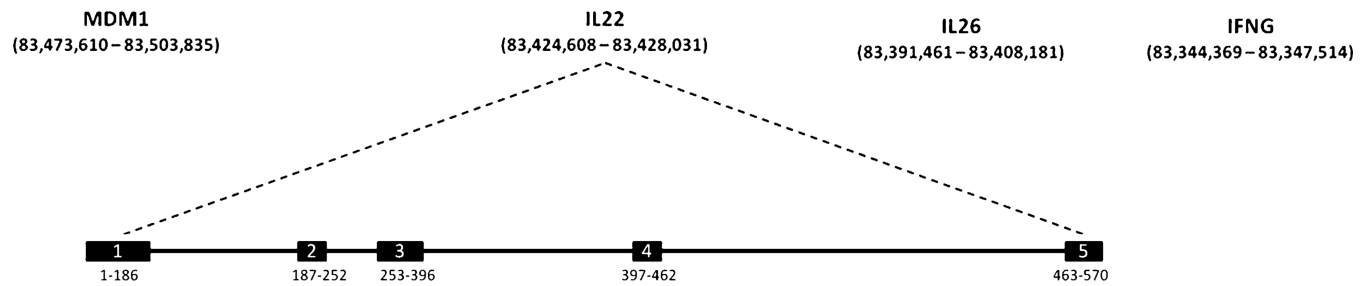


**FIGURE 7.**

Tree diagram depicting the relationship between the horse IL-22 protein sequence and IL-22 from selected other species. The tree was generated in MEGA5 using the neighbor-joining method and rooted on the horse. The optimal tree with the sum of branch length = 0.86793050 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale. Branch lengths (next to the branches) represent evolutionary distances computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 179 positions in the final dataset.

**FIGURE 8.**

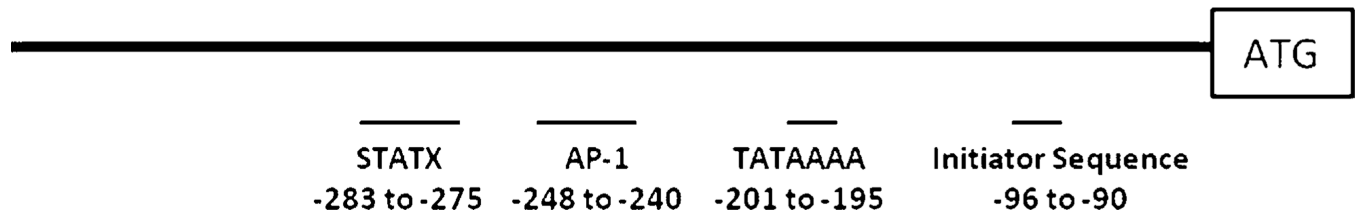
Predicted secondary structure of horse IL-22. The predicted amino acid secondary structure shows that horse IL-22 is primarily  $\alpha$ -helical as in other species. Cylinder,  $\alpha$ -helix; arrow,  $\beta$ -sheet; downward pointing arrow, turn; wavy line, coil. The predicted structure of the 3' extension is also  $\alpha$ -helical. Analysis performed with Geneious 5.0.4.



**FIGURE 9.**

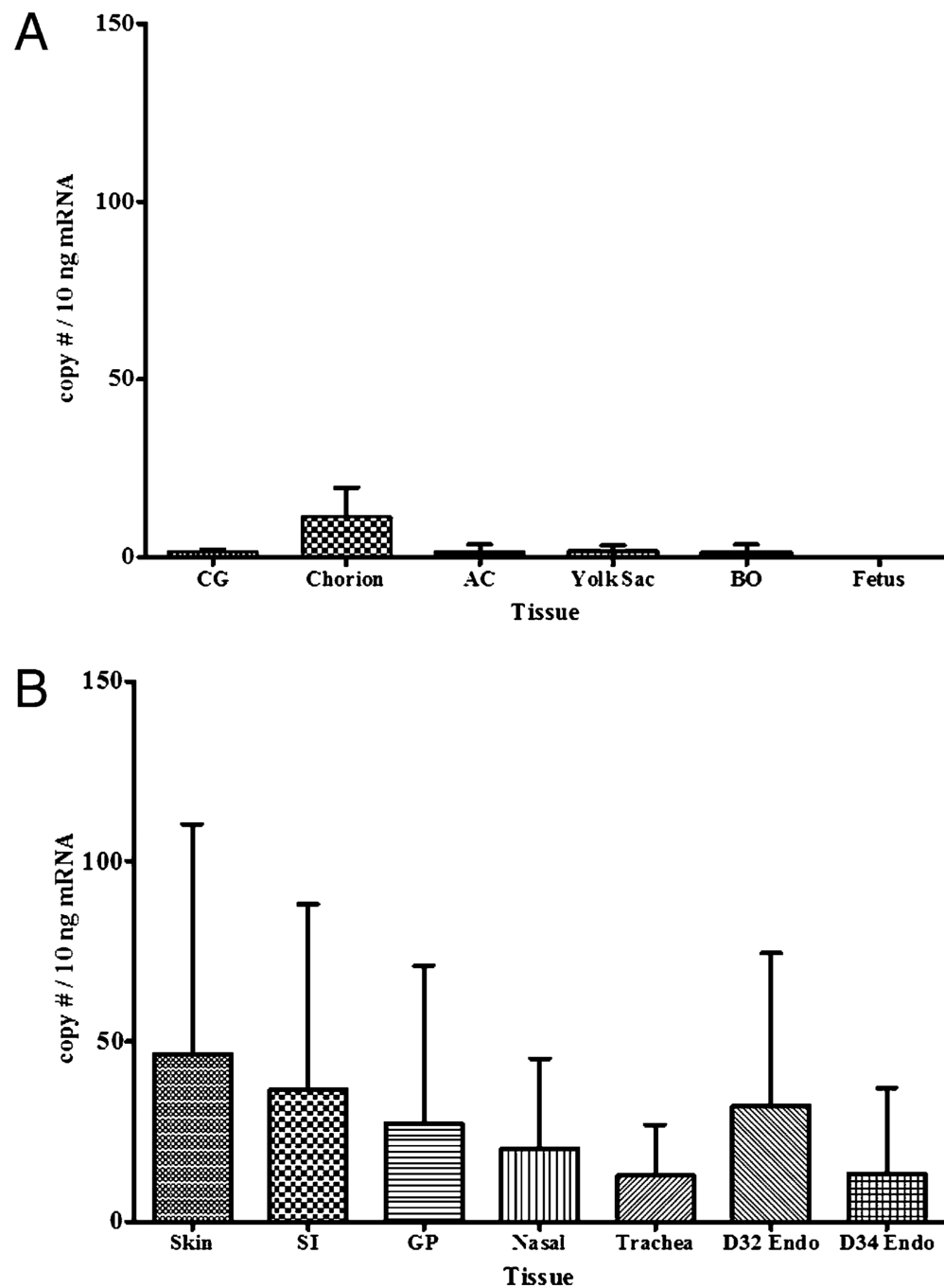
Exon structure and genomic landscape of IL-22 on equine chromosome 6. The horse IL-22 gene is located on equine chromosome 6 and contains five coding exons. This diagram shows the position of the IL-22 genomic sequence on chromosome 6 and distribution of the coding sequence across exons (*bottom numbers*). Neighboring genes include MDM1, IL-26, and IFNG, as are found in other species.





**FIGURE 10.**

Schematic of upstream regulatory elements in equine IL-22. This schematic shows putative regulatory elements identified using in silico analysis of the equine IL-22 promoter region. The sites are conserved with other mammalian species having various types of placentation, including the human, dog, pig, and cow. An identical initiator sequence is present in the horse, pig, and cow. The same motif is present in the human with a single nucleotide difference, and is not apparent in the dog.

**FIGURE 11.**

IL-22R1 expression in equine tissues. IL-22RA1 expression assessed by qRT-PCR in 3-d 34 equine conceptuses (**A**) and in other equine epithelial tissues (**B**). Epithelial samples included endometrium from days 32 ( $n = 2$ ) and 34 ( $n = 8$ ) of pregnancy, skin ( $n = 6$ ), nasal mucosa ( $n = 6$ ), tracheal mucosa ( $n = 6$ ), guttural pouch mucosa (GP,  $n = 4$ ), and small intestine (SI,  $n = 2$ ). Samples were run in triplicate.

**Table I**

Primer sequences used for amplification of equine products in RT-PCR assays

Gene	Reaction	Forward 5'–3'	Reverse 5'–3'
IL-22 <sup>a</sup>	RT-PCR	ATGGCCACCCTGCAGAAAT	TTAGTTATCCATTTCCAGCTTTTCTC
β <sub>2</sub> m	RT-PCR	TATGTCTCTGGGTTCCATCC	GATGCTGGTTAGAGGTCTCG
IL-22	qRT-PCR	CACAGATGTTCTGGCTCATTG	GTAAGGCTGGAATCGGTCAG
IL-22R1	qRT-PCR	TTCCAACCTGGTCCAAAGAG	CACCTGAGAAGGCGTAGGTC
IL-22R2	qRT-PCR	AGCTCACAGGGTCATTGAGG	CTCAGGCCGGTAGATTTCAG
CD3	qRT-PCR	GGCCTCATCCTGGCTATCAC	CCCAGATTCCGTGTAGTTTCTC
SCAMP3	qRT-PCR	CTGTGCTGGGAATTGTGATG	ATTCTTGCTGGGCCTTCTG

<sup>a</sup>Primer set used for cloning of the entire cDNA.

**Table II**

Expression array results for IL-22 and the IL-22Rs in CG, chorion, and lymphocytes

Gene	Tissue of Interest	Comparison Tissue	Array	Probes	Fold Change	p Value	Tissue of Interest Signal
IL-22	CG	Chorion	Cornell 8 × 15	Single	903.72	0.0007	12.23
			Agilent 4 × 44	Mean of 5	856.76	0.016	12.31
	Stimulated lymphocytes	Resting lymphocytes	Cornell 8 × 15	Single	628.36	0.02	14.23
			Agilent 4 × 44	Mean of 5	2029.14	0.044	14.31
IL-22R1	CG	Chorion	Cornell 8 × 15	Single	-1.38	0.02	8.03
			Agilent 4 × 44	Single	-1.49	0.03	8.88
	Stimulated lymphocytes	Resting lymphocytes	Cornell 8 × 15	Single	-1.13	NS	8.71
			Agilent 4 × 44	Single	-1.05	NS	8.78
IL-22R2	CG	Chorion	Cornell 8 × 15	Single	1.04	NS	3.70
			Agilent 4 × 44	Single	1.74	NS	4.29
	Stimulated lymphocytes	Resting lymphocytes	Cornell 8 × 15	Single	435.32	NS	11.33
			Agilent 4 × 44	Single	631.32	0.05	14.04

**Table III**

Amino acid sequence identity between equine IL-22 with and without the 3' extension and other species

	<i>Equus caballus</i> (Full Sequence) (%)	<i>Equus caballus</i> (Short Sequence) (%)
<i>Homo sapiens</i>	75	79
<i>Mus musculus</i>	69	73
<i>Canis familiaris</i>	76	80
<i>Bos taurus</i> (full sequence)	64	–
<i>Bos taurus</i> (short sequence)	–	66
<i>Sus scrofa</i> (full sequence)	73	–
<i>Sus scrofa</i> (short sequence)	–	73

Analysis performed with Geneious 5.0.4.