Fell Pony Syndrome: Characterization of Developmental Hematopoiesis Failure and Associated Gene Expression Profiles

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Fell Pony syndrome (FPS) is a fatal immunodeficiency that occurs in foals of the Fell Pony breed. Affected foals present with severe anemia, B cell lymphopenia, and opportunistic infections. Our objective was to conduct a prospective study of potential FPS-affected Fell Pony foals to establish clinical, immunological, and molecular parameters at birth and in the first few weeks of life. Complete blood counts, peripheral blood lymphocyte phenotyping, and serum immunoglobulin concentrations were determined for 3 FPS-affected foals, 49 unaffected foals, and 6 adult horses. In addition, cytology of bone marrow aspirates was performed sequentially in a subset of foals. At birth, the FPS-affected foals were not noticeably ill and had hematocrit and circulating B cell counts comparable to those of unaffected foals; however, over 6 weeks, values for both parameters steadily declined. A bone marrow aspirate from a 3-week-old FPS-affected foal revealed erythroid hyperplasia and concurrent erythroid and myeloid dysplasia, which progressed to a severe erythroid hypoplasia at 5 weeks of life. Immunohistochemical staining confirmed the paucity of B cells in primary and secondary lymphoid tissues. The mRNA expression of genes involved in B cell development, signaling, and maturation was investigated using qualitative and quantitative reverse transcriptase PCR (RT-PCR). Several genes, including CREB1, EP300, MYB, PAX5, and SPI1/PU.1, were sequenced from FPS-affected and unaffected foals. Our study presents evidence of fetal erythrocyte and B cell hematopoiesis with rapid postnatal development of anemia and B lymphopenia in FPS-affected foals. The transition between fetal/neonatal and adult-like hematopoiesis may be an important aspect of the pathogenesis of FPS.
Developmental Hematopoiesis Failure in Fell Pony Syndrome

MATERIALS AND METHODS

Blood and tissue samples. Samples were collected prospectively from two Fell Pony herds in the United States from FPS-affected (n = 3) and healthy unaffected (n = 49) Fell Pony foals at birth and up to the time of euthanasia for FPS-affected foals or at 5 months of life for healthy foals. The initial FPS-affected foal (FPS1) was submitted for euthanasia at 79 days of life. Peripheral blood was diluted when values were greater than the upper limit of the standard curve. Serum samples were diluted when values were greater than the upper limit of the standard curve. Serum samples were diluted when values were greater than the upper limit of the standard curve.

Flow cytometric analysis of lymphocyte surface markers. Isolated peripheral blood lymphocytes were tested for cell surface molecule expression using murine monoclonal antibodies for equine T and B cells and anti-canine parvovirus antibody as a negative control (CD3 [Equine Leukocyte Antigen Workshop (ELAW) II 98], CD4 [ELAW I 72], CD8 [ELAW I 72], CD8 [ELAW I 72], CD9-like [ELAW II 73], IgM [ELAW II 23], and MHC class II [ELAW II 43]) (17, 34, 44, 55). After cell washes and labeling with a fluorescein isothiocyanate (FITC)-conjugated secondary antimurine antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), leukocyte subpopulations were displayed in a dot plot and gated according to size based on forward light scatter (FSC) and granularity based on 90-degree side light scatter (SSC). Cells compatible with lymphocytes based on size and granularity were gated for analyses of percentages of positive cells.

Serum immunoglobulin concentrations. Posttuckle serum IgM and IgG concentrations were determined using commercially available radial immunodiffusion kits for horses (VMRD, Pullman, WA) per the manufacturer’s instructions. A standard curve was generated with the known concentrations of purified equine immunoglobulins provided in the kit and their respective precipitate diameters. The concentrations of IgM or IgG in each serum sample were determined by comparing the individual precipitate diameters to that of the standard curve. Serum samples were diluted when values were greater than the upper limit of the standard curve.

Immunohistochemistry of lymphoid tissues. Five-micrometer tissue sections were cut with a cryotome and placed on glass slides. The sections were fixed for 10 min in acetone at 4°C. Immunohistochemical labeling
was performed in a humidity chamber at room temperature, as previously described (18). Tris-buffered saline (TBS) was used for washes. Blocking steps included separate 15-min incubations with (i) 0.3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) in 0.1% sodium azide (Sigma, St. Louis, MO) and TBS and (ii) 10% normal goat serum in TBS. The tissues were incubated for 2 h with the following primary monoclonal antibodies: irrelevant monoclonal antibody mouse anti-canine parvovirus, mouse anti-horse B cells (IgM and CD19-like), and mouse anti-horse T cells (CD4, CD8) (38, 44, 55). The secondary antibody, a goat-anti mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.), was incubated for 30 min. Substrate solution was prepared from concentrated acetate buffer, 3-amino-9-ethylcarbazole (AEC) chromogen, and hydrogen peroxide (AEC staining kit; Sigma). Counterstaining was performed using hematoxylin for 1 min (Fisher Scientific).

RT-PCR, subtractive hybridization, and sequencing. RNA was isolated from snap-frozen bone marrow and spleen samples following homogenization with a QiAshredder (Qiagen, Valencia, CA) as directed by the RNAeasy kit instructions (Qiagen). One microgram of RNA was treated with DNase I (Invitrogen, Carlsbad, CA). cDNA synthesis reaction mixtures contained 1× Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) buffer, 5.5 mM MgCl2, 0.5 mM deoxynucleoside triphosphates (dNTPs), 2.5 μM oligo(dT) (Applied Biosystems, Foster City, CA), 0.4 U RNasin RNase inhibitor (Promega, Madison, WI), and 1 U Moloney murine leukemia virus reverse transcriptase (MuLV RT; Applied Biosystems); control samples did not receive M-MuLV RT. Amplification reaction mixtures contained 1× PCR buffer, 1.5 mM MgCl2, 0.25 mM dNTPs, 0.6 μM forward and reverse primers (Integrated DNA Technologies, Coralville, IA), and 2 U Taq polymerase (Invitrogen).

Based on the literature, we composed a list of genes that, if mutated or dysregulated, might play a role in the lack of B cell and erythrocyte development in foals with FPS. RT-PCR assays were designed for the following genes, which were sorted into four categories based on function: (i) genes known to be important for supporting hematopoiesis in the bone marrow, including IL-7 and its receptor (IL7R), FLT3 and its ligand (FLT3LG), KIT and its ligand (KITLG), and PTPN2; (ii) genes imperative for B lymphocyte development, including SPI1/PU.1, EBFP1, TCF3/E2A, PTPRC/B220, PAX5, CD19, and IGHM; (iii) genes critical for B lymphocyte function and signaling, including IGHG, CD79A, CD79B, BTK, POU2F1, POU2F2, POU2F1J, JAK1, JAK3, STAT5A, and TRAF2; and (iv) other genes identified by subtractive hybridization and literature searches, including hemoglobin genes, EP100, CREBBP, CREB1, and MYB (2, 5, 9, 11, 14, 15, 24, 25, 29, 30, 32–35, 37, 40, 41, 45, 48, 50, 52, 54, 56, 58, 59, 61, 71–77). RT-PCR amplification of the β-actin (ACTB) gene was included as a cDNA quality control.

Primers were designed from equine sequence databases (EST and genomic trace archives) or consensus sequences (see Table S1 in the supplemental material; the assembled genome sequence was not available at that time). Thermal cycling parameters were 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s, and 60°C for 30 s, and a final extension of 72°C for 10 min. Amplification products were run on 1% agarose gels and stained with ethidium bromide for visualization.

Quantitative real-time reverse transcriptase PCR was performed with 50 nanograms of RNA, 500 nM primer, and iScript one-step RT-PCR with SYBR green mix (Bio-Rad, Hercules, CA) in a CFX96 real-time PCR detection system (Bio-Rad). Reactions were performed in triplicate. Cycling parameters were as follows: 1 cycle of 50°C for 10 min, 1 cycle of 95°C for 5 min, and 40 cycles of 95°C for 10 s and then 60°C for 30 s followed by melt curve analysis. SYBR primers spanning intron/exon boundaries were designed with Beacon Designer 7.91 software (Premier Biosoft International, Palo Alto, CA) and were synthesized by Eurofins MWG Operon (Huntsville, AL) (see Table S1 in the supplemental material).

For absolute quantitation, RNA standard curves were prepared for each gene. Each target gene was amplified from healthy adult bone marrow cDNA (primers are listed in Table S1 in the supplemental material) (i)Proof polymerase; Bio-Rad), cloned (pET12 vector; Fermentas, Glen Burnie, MD), and sequenced at the Cornell University Life Sciences Core Laboratories Center Genomics Facility (Ithaca, NY). RNA transcription was performed on linearized plasmid DNA (Fermentas), purified (Zymo Research Corporation, Irvine, CA), and quantified (NanoDrop). Tenfold serial dilutions were made for the standard curve. SYBR primers were validated on the RNA standard curve. Data were analyzed with CFX Manager software (Bio-Rad) to determine numbers of mRNA transcripts. Due to the small sample size, no statistical comparisons were performed.

Subtractive hybridization was performed as directed by the instructions of a PCR-Select cDNA subtraction kit (Clontech, Mountain View, CA) in bone marrow samples from FPS2 and a Warmblood control foal. To obtain full-length transcripts for sequencing of selected genes, gene-specific cDNA synthesis was performed with the Superscript III first-strand synthesis system for RT-PCR (Invitrogen) and amplification was performed with iProof high-fidelity DNA polymerase (Bio-Rad). PCR products were purified and cloned, and at least 3 clones per gene were sequenced. Sequences were analyzed with VectorNTI (Invitrogen) software.

The nucleotide sequence accession numbers. Accession numbers of genes submitted to GenBank are listed in Table 1.

**RESULTS**

To assess the temporal pattern of anemia, hematocrits were determined prospectively and sequentially from Fell Pony foals. At birth, two FPS-affected foals had hematocrits below or within the low range of the unaffected foals, and all 3 FPS-affected foals became severely anemic by 3 to 6 weeks of life. Data are not available for one of the affected foals at birth (Fig. 1).

Total lymphocyte counts were also monitored in FPS-affected and unaffected Fell Pony foals to determine the presence of lymphopenia. At birth, FPS-affected foals had total lymphocyte counts comparable to those of age-matched healthy control foals (Fig. 2). The lymphocyte counts of two of the foals failed to increase with age. One affected foal had lymphocyte counts comparable to those of unaffected foals at 6 weeks of life.

We assessed the percentage of peripheral blood lymphocytes expressing the cell surface markers for B cells (CD19-like and IgM), T cells (CD3, CD4, CD5, and CD8), and the MHC class II

### TABLE 1 Genes of interest sequenced from FPS-affected Fell Pony foals

| Gene    | Sequence length (bp)$^{a}$ | Regions sequenced$^{b}$ | Sequence difference(s) | GenBank accession no. |
|---------|-----------------------------|--------------------------|------------------------|-----------------------|
| CREB1   | 1,412                       | Exon 1 through 3′ UTR    | None                   | JQ044378              |
| EP300   | 6,533                       | 5′ UTR to last exon      | 1 A to G and 1 T to C synonymous substitution | JN979561              |
| MYB     | 2,150                       | Exon 2 through 3′ UTR    | None                   | JQ070670              |
| PAX5    | 1,173                       | 5′ UTR through 3′ UTR    | 1 C to T synonymous substitution | JQ044379              |
| SPI1/PU.1| 820                        | 5′ UTR through last exon | None                   | JN979559              |

$^{a}$ Nucleotide sequence length excluding primers.

$^{b}$ UTR, untranslated region.
molecule in all lymphocytes using flow cytometry. FPS2 and FPS3 foals were born with a peripheral blood B cell subpopulation distribution (CD19-like \(^+\) IgM \(^+\)) comparable to those of unaffected foals and adult Fell Pony horses. However, a B cell lymphopenia developed rapidly with age in all 3 FPS-affected foals. The presence of B lymphopenia is a key feature of the FPS phenotype (67, 68).

The expression of MHC class II on lymphocytes in FPS2 and FPS3 was comparable to that of unaffected foals in the time points measured, whereas FPS1 had low expression by 6 weeks of life. Foals were sampled sequentially, although not all foals were sampled at each time point.

FPS-affected foals failed to increase in serum IgM concentrations with age, in contrast to unaffected Fell Pony foals (Fig. 4). The postsuckle serum IgM concentration in FPS2 in the first day of life was comparable to that in unaffected foals but decreased within the first month of life. Conversely, serum IgG concentrations remained above protective levels in FPS1 and FPS2 until euthanasia (1,600 mg/dl for both).

Sequential bone marrow aspirates were performed at 3, 5, and 8 weeks of life in FPS2, whereas a single marrow sample was collected from FPS3 at 6 weeks of life. Bone marrow aspirates were also collected from 6 unaffected Fell Pony foals between 1 and 6 weeks of life, with sequential aspirations at 2 and 6 weeks being performed for 1 foal. Bone marrow cytology results varied with time of sampling in the FPS-affected foals. When FPS1 was severely anemic, with a hematocrit of 13% at 3 weeks of life, there was an erythroid hyperplasia, with left-shifted maturation (increased proportion of immature precursors and relatively few stages seen beyond basophilic rubricytes) and concurrent dysplasia (characterized by multinucleation, abnormal nuclear shapes, syncytial formation, hypochromic nuclei, and apoptosis) (Fig. 5).

Phagocytosis of erythroid progenitors by macrophages was observed. Mild dysplasia in myeloid cells (hypersegmented neutrophils) was also evident. The morphological diagnosis was ineffective erythropoiesis with bilineage dysplasia. These morphological abnormalities in erythroid and myeloid progenitors were not observed in aspirates taken from unaffected Fell Pony foals. Sequential bone marrow aspirates revealed that the ineffective erythropoiesis progressed in the foal to a severe erythroid hypoplasia at 5 weeks of life (myeloid cell-to-erythroid cell ratio of 7.1 to 99:1; range in 5- to 6-week-old unaffected foals of 0.4 to 1.1:1), with only early-stage erythroid progenitors (prorubricytes and basophilic rubricytes, some of which were binucleate) being observed. Neutrophil hypersegmentation was still present. Similar results for erythroid and myeloid progenitors were obtained with marrow collected from FPS3 at 6 weeks of life. A bone marrow aspirate and a section of bone marrow taken at necropsy at 8 weeks of life from FPS2 revealed a hypoplastic marrow consisting of myeloid precursors and erythropagocytic histiocytes, with no erythroid precursors identified, compatible with a pure red cell aplasia.

To characterize the distribution of lymphocyte subpopulations and organization of lymphoid tissues, samples collected at necropsy from FPS1 and FPS2 were assayed using immunohistochemical staining (Fig. 6). In contrast to what was observed with the control samples from age-matched healthy Warmblood foals, two equine B cell markers (CD19-like and IgM) revealed the absence of B cells in the bone marrow and lymph nodes of FPS-affected foals and a paucity of B cells in the spleen, with the absence of germinal centers. The distributions of T cells (CD4 and CD8 markers) were similar in the lymph nodes and spleens of FPS-affected and healthy foals; however, T cells were absent from the bone marrow of FPS1.

The fact that B cells were absent in the bone marrow led us to test for the mRNA expression of genes known to be involved in B cell hematopoiesis and development; RT-PCR was initially used to screen for absent gene expression. To account for differences due to developmental stages, FPS1 and FPS2 lymphoid tissue samples...
FIG 3 Sequential flow cytometric assessment of peripheral blood lymphocyte subpopulation distributions in FPS-affected and unaffected Fell Pony foals from birth up to 21 weeks of life and in adult Fell Pony horses. Lymphocyte subpopulations are expressed as percentages of cells positive for the selected marker within the defined lymphocyte gate in flow cytometric dot plot analyses. FPS-affected foals ($n = 2$; data are not available for FPS1) were born with a peripheral blood B cell subpopulation distribution (CD19-like $^+$ IgM $^+$) comparable to those of unaffected foals ($n = 46$) and adult Fell Pony horses ($n = 6$). However, a B cell lymphopenia developed rapidly with age in all 3 FPS-affected foals. The expression of MHC class II on lymphocytes in 2 FPS-affected foals was comparable to that of unaffected foals in the time points measured, whereas FPS1 had low expression by 6 weeks of life. The distribution of CD3 $^+$, CD4 $^+$, CD5 $^+$, and CD8 $^+$ T cell subpopulations in all FPS-affected foals was comparable to that of unaffected foals. Foals were sampled sequentially, although not all foals were sampled at each time point.
along with age-matched healthy Warmblood control samples were assayed by RT-PCR. mRNA expression was detected in both FPS-affected and healthy Warmblood foals for IL-7 and its receptor (IL7R), FLT3 and its ligand (FLT3LG), KIT and its ligand (KITLG), PTPN2, SPI1/PU.1, TCF3/E2A, PTPRC/B220, CD19, and IGHM (Fig. 7). No mRNA for EBF1 was detected in the spleen of FPS2, but transcripts were detected in the bone marrow of FPS1 and FPS2. Remarkably, PAX5 mRNA expression was not detected in either the bone marrow or spleens of FPS1 and FPS2, and thus the entire coding sequence of PAX5 was determined from an age-matched healthy Warmblood foal and FPS2. Only one nucleotide difference was identified in the PAX5 sequence from FPS2 (Table 1); yet, this polymorphism did not cause an amino acid change. Since this work was completed, the annotated equine genome sequence has become available. The predicted equine PAX5 mRNA sequence is identical to the sequence that we determined from the healthy Warmblood foal. Although mRNA expression was detected, the coding sequence of SPI1 was specifically determined because SPI1/PU.1 mutants have impaired erythroblast maturation and lack B lymphocytes (61). No sequence differences were identified between FPS-affected and healthy foals (Table 1), but the amino acid sequence of the KIX domain obtained from FPS1 and FPS2 was comparable to the numbers in age-matched healthy Warmblood foals (16,967 and 3,030 copies). A similar pattern of transcript numbers (272 and 615 copies, respectively), in contrast to transcript numbers in age-matched healthy Warmblood foals (16,967 and 3,030 copies). A similar pattern of transcript numbers (272 and 615 copies, respectively), in contrast to transcript numbers in age-matched healthy Warmblood foal bone marrow or spleen tissues (Fig. 7).

Pursuing a candidate gene approach based on published literature, we investigated the expression of genes involved in the phenotype of mice with mutations in the EP300 KIX domain (EP300, CREBBP, CREB1, and MYB) (Fig. 7). mRNA expression was detected for EP300 and CREBBP genes in FPS1, FPS2, and healthy Warmblood foals using RT-PCR. The mRNA expression of CREB1 was weak in the bone marrow of all foals tested and was absent (FPS1) or weak (FPS2) in the spleens of affected foals in comparison to healthy foals. MYB mRNA expression appeared to be absent from FPS1 and FPS2 tissues, unlike in the bone marrow and spleens of healthy foals. Due to the importance of the EP300 KIX domain in erythropoiesis and B cell lymphopoiesis (30), we next sequenced the EP300 transcript from FPS2 and a healthy Warmblood foal, although primers flanking the entire coding sequence could not be designed (lacking the last 1,130 bases of the 7,263-nucleotide coding sequence). Two synonymous substitutions were identified between FPS-affected and healthy foals (Table 1), but the amino acid sequence of the KIX domain obtained from these foals was identical to the human EP300 KIX domain sequence and to the predicted equine EP300 sequence.

Quantitative RT-PCR was undertaken for the three genes with subjective differences in mRNA expression identified by standard RT-PCR: PAX5, CREB1, and MYB (Fig. 8). PAX5 mRNA transcript numbers were low in the bone marrow of FPS1 and FPS2 (272 and 615 copies, respectively), in contrast to transcript numbers in age-matched healthy Warmblood foals (16,967 and 3,030 copies). A similar pattern of PAX5 mRNA expression was observed in the spleen. The numbers of CREB1 transcripts detected in FPS1 and FPS2 were comparable to the numbers in age-matched healthy Warmblood control foal tissues. Trancript numbers of MYB were low in FPS2 bone marrow, in contrast only

![Fig 4](https://example.com/fig4.png) Sequential serum IgM concentrations in FPS-affected and unaffected Fell Pony foals from birth (postsuckle) to 18 weeks of life and in adult Fell Pony horses. No increase in IgM concentrations with age was measured in postsuckle serum samples of FPS-affected foals (n = 3), unlike in unaffected Fell Pony foals (n = 35). Results for 2 adult Fell Pony horses overlap. Foals were sampled sequentially, although not all foals were sampled at each time point.

![Fig 5](https://example.com/fig5.png) Photomicrographs of Wright’s-stained smears of a bone marrow aspirate collected from an FPS-affected Fell Pony foal at 3 weeks of life. Early erythroid progenitors (prorubricytes, basophilic rubricytes) dominate, with evidence of erythroid and myeloid dysplasia. (A) Abnormal nuclear shapes (lobulation, blebbing) in metarubricytes; (B) multinucleation or syncytial formation in a basophilic rubricyte (arrow); (C) binucleate prorubricyte (arrow), abnormal nuclear shape in a metarubricyte (arrowhead), and hypersegmented neutrophil. Scale bar = 10 μm.
onset may be significant, since the disease manifests during the
node and spleen are similar in all foals; however, T cells were not present in the bone marrow of FPS1. Centres revealed a paucity of B cells in FPS-affected foals, in contrast to results for the age-matched healthy foal spleen. The distributions of T cells in the lymph node and spleen are similar in all foals; however, T cells were not present in the bone marrow of FPS1.

FIG 6 Immunohistochemical staining for equine-specific lymphocyte markers for B cells (CD19-like and IgM) and T cells (CD4 and CD8) in lymphoid tissues from FPS-affected Fell Pony foals and a healthy Warmblood foal. Lymphoid tissues were collected at necropsy and stained for lymphocyte markers using immunohistochemistry. FPS1 was submitted to euthanasia at the age of 5 weeks and FPS2 at 7 weeks; tissues from a 4-week-old Warmblood foal (shown) and an 8-week-old foal (not shown) were used as controls. No B cells were detected in the bone marrow and lymph nodes of FPS-affected foals. Splenic germinal centres were paucity of B cells in FPS-affected foals. Splenic germinal centres revealed a paucity of B cells in FPS-affected foals. The distributions of T cells in the lymph node and spleen are similar in all foals; however, T cells were not present in the bone marrow of FPS1.

to the transcript numbers in FPS1 (2.1 \( \times 10^{10} \) versus 4.5 \( \times 10^{10} \) copies) and a healthy Warmblood control foal (1.1 \( \times 10^{10} \) versus 2.3 \( \times 10^{10} \) copies) tissues. We also sequenced sections of the CREB1 and MYB genes in FPS1 and FPS2 spleen tissues and an age-matched healthy Warmblood foal. No sequence differences from the healthy foal sequence or the GenBank predictions were identified in the coding sequence of either CREB1 or MYB (Table 1).

**DISCUSSION**

This study describes the first prospective clinical and molecular investigation of Fell Pony syndrome from birth to euthanasia, including sequential collection of bone marrow samples for cytologic analysis of FPS-affected and unaffected Fell Pony foals. The novel findings about the temporal distribution of events that lead to anemia and B cell lymphopenia described in this study include the following: (i) FPS-affected foals may be born with erythroid precursors in the bone marrow and hematocrits and peripheral blood B cell distributions equivalent to those of healthy unaffected foals, suggesting a productive hematopoiesis during fetal life, (ii) they exhibit perinatal development of a progressive B lymphopenia and anemia, (iii) there is bone marrow cytologic evidence of initial erythroid hyperplasia with dysplasia, followed by a rapid and progressive hypoplasia and aplasia, along with anemia, and (iv) they have concomitant mild myeloid dysplasia. Despite the evidence that B cells seem to be produced during fetal life, B cells were not found in the bone marrow in postmortem samples, and this absence was accompanied by low PAX5 gene expression in the bone marrow. All together, our data suggest a limited but detectable B cell and red cell poiesis during fetal life that is not sustained after birth in FPS-affected foals.

The prospective aspect of our study was essential for revealing the morphological abnormalities of erythroid and myeloid precursors, which were likely missed by the previous studies due to the timing of sample analysis and technique used (histology versus cytology) (28, 57, 60). We hypothesize that the timing of disease onset may be significant, since the disease manifests during the transitioning period from fetal/neonatal to foal stages; perhaps bone marrow function is more critical in that period than during fetal life. Indeed, there remains an important question about the importance of the liver versus bone marrow as hematopoietic organs during the fetal life of FPS-affected individuals; one may speculate that the red cells and B cells detected in the peripheral blood of FPS-affected equine neonates may have been developed in the fetal liver, with or without limited bone marrow contributions. All together, the erythroid hypoplasia/dysplasia/aplasia, B cell lymphopenia/depletion, and myeloid dysplasia suggest a common underlying defect affecting hematopoietic stem cell lineage differentiation rather than three separate abnormalities in erythroid, lymphoid, and myeloid cells. Cytologic examination of bone marrow aspirates from additional FPS-affected foals taken in the first week of life would help to further elucidate the cell differentiation defect. Similar morphological abnormalities in erythropoiesis are observed in other congenital hematopoietic disorders in humans and animals, including congenital dyserythropoietic anemia and Fanconi syndrome (8, 26). However, these syndromes lack the concurrent B cell lymphopenia.

The inability of FPS-affected foals to produce IgM in the critical phase of primary pathogen exposure indicates an intrinsic humoral dysfunction. Equine colostrum is a poor source of IgM and has a half-life of 5 to 8 days; therefore, maternally derived IgM is depleted in foals by 4 weeks of life (39). In the absence of endogenous production, serum IgM concentrations decrease over time, as we observed in the FPS-affected but not in the unaffected Fell Pony foals. The equine fetus produces IgM during gestation and may be born with serum IgM concentrations of 10 to 25 mg/dl (66). Of note, FPS1 postsuckling serum IgM concentrations close to birth were within the range observed for healthy Fell Pony foals, likely due to colostral transfer. Without analysis of presuckle samples, we cannot rule out the possibility that affected foals also have a failure of fetal IgM production. In contrast to IgM, IgG has a long half-life of 23 to 30 days; thus, the normal serum IgG concentra-
tions measured in older affected foals at the time of death or euthanasia were likely due to the persistence of circulating maternal antibodies (39). It is likely that serum IgG concentrations would have progressively decreased in affected foals had they survived longer.

Absolute lymphopenia in FPS-affected foals likely reflects B cell lymphopenia in peripheral blood and primary and secondary lymphoid tissues. It also may be a consequence of T cell lymphopenia or dysfunction. The presence of T cell lymphopenia is supported by our data showing a lack of increases in total lymphocyte counts with age in 2 FPS-affected foals and the absence of T cells in the bone marrow of 1 FPS-affected foal. Also, affected foals typically have small thymi and suffer from opportunistic infections with cryptosporidia and adenovirus, despite normal (colostrum-derived) serum IgG concentrations (23). Murine studies have demonstrated that T cells are important in resisting primary and secondary Cryptosporidium muris infection and in clearing the pathogen (27, 47). CD4<sup>+</sup> and CD8<sup>+</sup> T cells are also imperative in the control of adenovirus infection (20, 64, 65). Our study did not document a failure in the expression of MHC class II molecules in lymphocytes of FPS-affected foals, reported in other studies; yet, such finding would further support abnormal lymphocyte development (3, 23, 43). Further studies focused on T cell differentiation and function in FPS-affected foals are necessary to confirm the involvement of yet another cell lineage in this syndrome, the T cells.

Our RT-PCR survey assessed the mRNA expression of genes involved in hematopoiesis, B lymphocyte development, function, and signaling and genes with potential involvement in FPS, based on the literature. Results revealed that most of these genes, with the exception of PAX5 and, potentially, MYB, were expressed in FPS-affected and healthy foals. PAX5 is essential for B lineage
commitment and development, as well as in suppressing alternative cell fates in the bone marrow; the expression of PAX5 is also essential for B cell survival peripheral tissues (51, 53). PAX5 mRNA expression measured by standard and quantitative RT-PCR was low in both the bone marrow and spleens of FPS-affected foals but not in control foals; yet, downstream PAX5-dependent CD19 and CD79A genes were measured in FPS-affected foals using standard RT-PCR (16, 54, 70). We hypothesize that the detected CD19 and CD79A mRNA expression results from the persistence of a limited number of B cells developed during fetal life. MYB expression is required for definitive erythropoiesis during fetal life, and it dictates both T cell numbers and early T cell differentiation events (13, 59, 69). Our results are inconclusive for an abnormal expression of MYB in FPS-affected foals, and additional samples would be necessary to determine its role in FPS.

To date, it is difficult to reconcile our findings, the progression of FPS, or the manifestation of postnatal anemia and B lymphopenia with the nonsynonymous SLC5A3 mutation reported to be predictive of disease status (22). The SLC5A3 P446L substitution was proposed to affect substrate binding, which would presumably result in the loss of SLC5A3 function (22). However, the phenotype of SLC5A3 null mice includes prenatal skeletal development defects, postnatal bone formation defects, and death shortly after birth due to hypoventilation (4, 10). If indeed the SLC5A3 mutant is not functional, it may be consistent with the peripheral ganglionopathy observed in FPS-affected foals and the requirement for myo-inositol for the development of peripheral nerves (7). Human genome-wide association studies have identified associations between SLC5A3 variations and the risk of coronary artery disease and early-onset myocardial infarction (31, 42). No impairments in cardiac development or function have been reported in FPS-affected foals. It is noteworthy that the tissue distribution of SLC5A3 is uncertain and that a causal relationship between the SLC5A3 mutation and Fell Pony syndrome has not been established. Therefore, it remains possible that other mutations in that genomic region may be responsible for the phenotype of Fell Pony syndrome, which warrants continued studies of candidate genes.

In summary, our study brings evidence of fetal erythrocyte and B cell hematopoiesis, with rapid postnatal development of anemia and B lymphopenia, in FPS-affected foals. Sequential cytologic analyses of bone marrow aspirates collected close to birth and in the first few weeks of life provided valuable information about the rapid progression of the syndrome, allowed us to observe a failure in hematopoiesis, introduced the finding of myeloid dysplasia, and highlighted the importance of early testing before clinical signs are fully manifested. Most essential B cell differentiation genes and genes associated with erythropoiesis are expressed in affected foals, and a potential common hematopoietic genetic defect is still under investigation. Further, this primary immunodeficiency is a unique natural model for studying mammalian hematopoiesis and developmental regulation in the perinatal period. The transition between fetal/neonatal and adult-like hematopoiesis may be an important aspect of the pathogenesis of FPS.

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