Fell Pony Syndrome: Characterization of developmental hematopoiesis failure and associated gene expression profiles

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Running title: Developmental hematopoiesis failure in Fell Pony Syndrome
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 comparable hematocrit and circulating B cell counts to 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 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.
INTRODUCTION

Fell Pony Syndrome (FPS) is a fatal disorder of young foals of the Fell Pony breed. FPS-affected foals are born healthy but rapidly fail to thrive, and experience severe anemia and lymphopenia within several weeks of life. Pedigree analysis of the breed suggests that FPS may have an autosomal recessive inheritance, and a recent genome-wide association study identified a mutation associated with the syndrome on chromosome ECA26 (22). The mechanistic implications of this mutation have not been resolved. In the absence of effective therapies, careful breeding management would be necessary to decrease FPS incidence. This is of particular importance because the Fell Pony breed is already considered threatened due to the small population size globally.

In 2006, our laboratory reported the first case of Fell Pony Syndrome in the United States, along with others in the Czech Republic and Netherlands (6, 23, 28). Most recently, a case of the same syndrome was reported in a Dales pony, a breed that also originated in England (21).

Fell Pony Syndrome was initially described in the United Kingdom in 1998 (60). The fatal illness became clinically apparent in foals within one month and death occurred generally by three months of life. Clinical and post-mortem examinations revealed a syndrome comprised of anemia, immunodeficiency, and peripheral ganglionopathy. Further findings included small numbers of late erythroid precursors in the bone marrow, small thymi, lack of secondary lymphoid follicles or germinal centers, and absence of plasma cells. Opportunistic infections caused by cryptosporidia and adenovirus were observed, and suggested an immunodeficiency condition. The FPS-affected foals were both male and female, no environmental cause could be identified,
and the findings described were not consistent with other known causes of anemia in foals. The authors concluded that a genetic defect was responsible for this syndrome. A second clinical report followed with similar findings (57). Bell and co-workers (3) described that FPS-affected foals had fewer total lymphocyte counts in comparison to healthy foals, and the distribution of CD4+ and CD8+ T lymphocytes appeared normal. The expression of major histocompatibility complex class II (MHC class II) molecule was decreased in peripheral blood lymphocytes in comparison to healthy foals. The B lymphopenia was confirmed in 2 subsequent studies (67, 68). A functional consequence of B lymphopenia is the inability to produce immunoglobulins (Igs), however several studies did not find decreased serum IgG levels in FPS-affected foals most likely due to the presence of maternal colostral antibodies (57, 60, 68). Nevertheless, ELISA-based quantification revealed significant reductions in serum IgM concentrations (a parameter not confounded by maternally-derived antibodies), and the absence of a primary humoral immune response in FPS-affected foals (67).

During fetal life, B cells are produced in the yolk sac, liver and bone marrow at different stages of gestation (46, 63). In the mouse, it has been demonstrated that the fetal liver is the major hematopoietic organ during embryonic development until birth, when the bone marrow becomes the major blood cell producing organ (12). In contrast, in fetal pigs, B cells are detected in the bone marrow and spleen approximately two weeks after their appearance in the liver, by day 45 of the 114 day gestation (62). Studies in our laboratory have demonstrated that B lymphopoiesis and Ig gene recombination are active during equine fetal life, similar to B lymphopoiesis in the fetal pig (66). At 90–120 days (approximately one-third of the 330–340 day gestation), genes
essential for B cell development including CD20, CD21, CD22, CD27, CD40, CD40LG, PTPRC/B220, CD79A, CD79B, RAG-2, IGHM, IGHD, IGHA, IGHG isotypes, and the lambda light chain IGLC are expressed in the liver, bone marrow, and spleen. 

B lymphocytes and erythrocytes are the most severely affected cell populations in FPS, and both undergo critical developmental stages in the bone marrow. The condition may be caused by independent or common genetic abnormalities that affect both cell lines. Our hypothesis is that FPS-affected foals lose expression or function of a gene essential to hematopoiesis. To our knowledge, there have been no reports in humans with a similar clinical course of disease. Mouse models that resemble the condition in FPS-affected Fell Pony foals include SPI1/PU.1 and EP300KIX mutants. SPI1/PU.1 mutants die during late gestation or shortly after birth with impaired erythroblast maturation and lack of B lymphocytes (1, 36, 49, 61). However, macrophage, neutrophil, and T cell lineages are also affected in SPI1/PU.1 mutants. Mice with mutations in the EP300KIX domain that diminish binding to CREB1 and MYB proteins exhibit anemia, B cell deficiency, thymic hypoplasia, megakaryocytosis, and thrombocytosis (30).

In most FPS-affected foals, the syndrome is not apparent at birth but progresses rapidly, and anemia and B cell lymphopenia are already present by the time of diagnosis, limiting the amount of information that can be obtained from absent cells. The sentinel case identified at Cornell University Hospital for Animals led to this prospective study of Fell Pony foals born on the same farm (23). An FPS-affected half-sibling to the index case was diagnosed, providing a unique opportunity to assess the progression of FPS with longitudinal and post-mortem samples for hematological, immunological and...
molecular gene expression studies. For the first time, we were able to measure normal concentrations of B cells and erythrocytes in peripheral blood of FPS-affected foals at birth, and to document the progressive and rapid loss of both cell types within the first few weeks of life. In addition, evaluation of bone marrow aspirates alluded to the importance of the transition period between fetal/neonatal and adult-like differentiation of erythrocytes and B cells, and the manifestation of disease. Our studies add novel clinical and molecular information about the progressive depletion of B cells and erythrocytes in FPS-affected foals.
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 or 5 months of life, respectively. The initial FPS-affected foal (FPS1) was submitted for euthanasia at 39 days of life (23) and a second FPS-affected foal identified in this study (FPS2) died at 51 days of life. Several years after this study was initiated, a third FPS-affected foal was identified (FPS3), which was submitted for euthanasia at 79 days of life. Peripheral blood was collected via jugular venipuncture into vacutainer tubes containing heparin, EDTA, or no additives for lymphocyte phenotyping, hemograms, and serum immunoglobulin concentrations, respectively. Peripheral blood lymphocytes were isolated from heparinized venous samples using methods described previously (19). The isolated cells were processed for immunophenotyping using flow cytometry and the excess were snap frozen for gene expression analyses. Serum was separated from blood cells using centrifugation (3,000 g for 15 minutes) and stored at –20°C. Not all analyses were performed on each sample and some foals were sampled repeatedly over time.

Bone marrow aspirates and core biopsies were obtained from the wing of the ileum from 2 FPS-affected and 2 unaffected Fell Pony foals. Bone marrow, spleen, and mesenteric lymph node samples were collected from FPS1 and FPS2 foals immediately after euthanasia and snap-frozen in liquid nitrogen or preserved in Tissue-Tek O.C.T. Compound (optimal cutting temperature medium, SakurFinetek U.S.A., Inc., Torrance, CA), and stored at –80°C until use. No unaffected, healthy Fell Pony foal was submitted...
to euthanasia during the study period; this breed is under danger of extinction, and
dividuals have high economic value. Therefore, archived tissue samples from 2 age-
matched healthy non-Fell Pony breed foals (Warmblood, at 4 and 8 weeks of life) were
used as controls. These experiments were approved by the Cornell University Center
for Animal Resources and Education and Institutional Animal Care and Use Committee
for the use of live vertebrates in research.

Hemogram analysis and bone marrow aspirate cytology

EDTA-anticoagulated peripheral blood was processed with an automated
hematology analyzer (ADVIA 120 hematology analyzer, Bayer Corporation, Tarrytown,
NJ) in the Clinical Pathology Laboratory at Cornell University. The analyzer provides
total leukocyte, erythrocyte and platelet counts, and measurements of erythrocyte and
platelet size (mean corpuscular volume and mean platelet volume, respectively) and
erthrocyte hemoglobin content (mean corpuscular hemoglobin concentration). A
manual leukocyte differential count (100 leukocytes) and assessment of cell
morphologic features were performed on Wright's-stained peripheral blood smears.
Smears of the bone marrow were prepared immediately after aspiration and
stained with Wright's stain before examination by one clinical pathologist (TS).
Assessment included estimates of cellularity and megakaryocyte number, calculation of
a myeloid to erythroid ratio based on a 200 differential cell count, percentage of
lymphocytes and plasma cells, and examination of morphologic features of
hematopoietic cells.
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 [ELAW II # 98], CD4 [ELAW I # 72], CD5 [ELAW I # 53], CD8 [ELAW I # 12], CD19-like [ELAW II # 73], IgM [ELAW II # 23], and MHC class II [ELAW II # 43]) (17, 38, 44, 55). After cell washes and labeling with a fluorescein isothiocyanate (FITC)-conjugated secondary anti-murine 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 percentage positive cell analyses.

Serum immunoglobulin concentrations

Post-suckle serum IgM and IgG concentrations were determined using commercially available radial immunodiffusion kits for horses (VMRD, Pullman, WA) per 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 serum IgM or IgG in each 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-micron tissue sections were cut with a cryotome and placed on glass slides. The sections were fixed for 10 minutes 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 minute incubations with: a) 0.3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) in 0.1% sodium azide (Sigma, St. Louis, MO) and TBS; and b) 10% normal goat serum in TBS. The tissues were incubated for 2 hours 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 minutes. Substrate solution was prepared from concentrated acetate buffer, 3-amino-9-ethylcarbazole (AEC) chromagen, and hydrogen peroxide (AEC staining kit, Sigma). Counterstaining was performed using hematoxylin for 1 minute (Fisher Scientific).

Reverse transcriptase polymerase chain reaction (RT-PCR), subtractive hybridization, and sequencing

RNA was isolated from snap-frozen bone marrow and spleen samples following homogenization by QIAshredder (Qiagen, Valencia, CA) as directed by the RNeasy kit (Qiagen). One microgram of RNA was treated with DNase I (Invitrogen, Carlsbad, CA). Complementary DNA synthesis reactions contained 1X M-MuLV RT buffer, 5.5 mM
MgCl₂, 0.5 mM dNTPs, 2.5 μM oligo(dT) (Applied Biosystems, Foster City, CA), 0.4 U RNasin Ribonuclease 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 reactions contained 1X PCR buffer, 1.5 mM MgCl₂, 0.25 mM dNTPs, 0.6 μM forward and reverse primer (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 FPS. RT-PCR assays were designed for the following genes, which were sorted into four categories based on function: 1) genes known to be important for supporting hematopoiesis in the bone marrow including IL7 and its receptor IL7R, FLT3 and its ligand FLT3LG, KIT and its ligand KITLG, and PTPN2; 2) genes imperative in B lymphocyte development including SPI1/PU.1, EBF1, TCF3/E2A, PTPRC/B220, PAX5, CD19, and IGHM; 3) genes critical for B lymphocyte function and signaling including IGHG, CD79A, CD79B, BTK, POU2F1, POU2F2, POU2AF1, JAK1, JAK3, STAT5A, and TRAF2; and 4) other genes identified by subtractive hybridization and literature searches including hemoglobin genes, EP300, 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 (Supplementary Table 1; the assembled genome sequence was not available at that time). Thermal cycling parameters were 95°C for 5 minutes; 35 cycles of 95°C for 60 seconds, 58°C for 60 seconds, 72°C for 30
seconds; and a final extension of 72°C for 10 minutes. 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 of 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: 1 cycle of 50°C for 10 minutes, 1 cycle of 95°C for 5 minutes, 40 cycles of 95°C for 10 seconds then 60°C for 30 seconds, 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) (Supplementary Table 1).

For absolute quantitation, RNA standard curves were prepared for each gene. Each target gene was amplified from healthy adult bone marrow cDNA (primers in Supplementary Table 1; iProof polymerase, Bio-Rad), cloned (pJET1.2 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) and purified (Zymo Research Corporation, Irvine, CA) and quantified (NanoDrop). Ten-fold 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 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, cloned, and at least 3 clones per gene were sequenced. Sequences were analyzed with VectorNTI (Invitrogen) software and submitted to GenBank (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 is not available for one of the affected foals at birth (Figure 1).

Total lymphocyte counts were also followed in FPS-affected and unaffected Fell Pony foals to monitor for the presence of lymphopenia. At birth, FPS-affected foals had comparable total lymphocyte counts to age-matched healthy control foals (Figure 2). Two of the foals failed to increase lymphocyte counts with age. One affected foal had comparable lymphocyte counts to 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 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 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 3 foals was comparable to that of unaffected foals in the time points measured, whereas FPS1 had low expression by 6 weeks of life. The latter observation was not due to poor reagent recognition for individual polymorphic molecules because monocytes from the same blood samples of FPS-affected foals labeled positively with appropriate mean fluorescence intensity (data not shown). The
distribution of CD3+, CD4+, CD5+ and CD8+ T cell subpopulations in FPS-affected foals was comparable to unaffected foals (Figure 3).

FPS-affected foals failed to increase in serum IgM concentrations with age in contrast to unaffected Fell Pony foals (Figure 4). Post-suckle serum IgM concentration in FPS2 in the first day of life was comparable to unaffected foals, but decreased within the first month of life. Conversely, serum IgG concentrations remained above protective levels in FPS1 and FPS2 foals 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 aspirates at 2 and 6 weeks being performed in 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, hypocromic nuclei and apoptosis) (Figure 5). Phagocytosis of erythroid progenitors by macrophages was observed. Mild dysplasia in myeloid cells (hypersegmented neutrophils) was also evident. The morphologic diagnosis was ineffective erythropoiesis with bilineage dysplasia. These morphologic 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 to erythroid ratio of 7.1-99:1; range in 5 to 6-week-old unaffected foals of 0.4-1.1:1), with only early stage erythroid progenitors (prorubricytes and basophilic rubricytes, some of which were binucleate) being observed. Neutrophil hypersegmentation was still present. Similar findings in erythroid and myeloid progenitors were observed in marrow collected from FPS3 foal 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 foal revealed a hypoplastic marrow consisting of myeloid precursors and erythrophagocytic 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 foals were assayed using immunohistochemical staining (Figure 6). In contrast to the control sample 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 absence of germinal centers. The distribution of T cells (CD4 and CD8 markers) was similar in the lymph node and spleen of FPS-affected and healthy foals; however, T cells were absent in the bone marrow in FPS1 foal.

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 affected foal lymphoid tissue samples were assayed by RT-PCR along with age-matched healthy Warmblood control...
samples. Messenger RNA expression was detected in both FPS-affected and healthy Warmblood foals for *IL7* 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* genes (Figure 7). No mRNA for *EBF1* was detected in the spleen of FPS2 foal, but transcripts were detected in the bone marrow of FPS1 and FPS2 foals. Remarkably, *PAX5* mRNA expression was not detected in either the bone marrow or spleen of FPS1 and FPS2 foals, 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 foal (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 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 an FPS-affected and a healthy Warmblood foal (Table 1). The sequence predicted from the horse genome published more recently is also identical to the sequences we obtained. The mRNA expression for genes essential to B lymphocyte function and signaling, *IGHG*, *CD79A*, *CD79B*, *BTK*, *POU2F1*, *POU2F2*, *POU2AF1*, *JAK1*, *JAK3*, *STAT5A*, and *TRAF2*, was detected in bone marrow and spleen from FPS1, FPS2, and the healthy Warmblood foals.

To identify differentially expressed genes in an unbiased manner, subtractive hybridization was undertaken using samples from FPS2 and an age-matched healthy
Warmblood foal. Five genes that differed in mRNA expression levels were detected with this method: Ig heavy and light chain, hemoglobin alpha (HBA), hemoglobin beta (HBB), and eukaryotic elongation translation factor 1 alpha 1 (EEF1A1). The expression of HBA, HBB, and EEF1A1 was further investigated by RT-PCR, and detected in FPS1, FPS2, and healthy Warmblood foal bone marrow or spleen tissues (Figure 7).

Pursuing a candidate gene approach based on published literature, we investigated expression of genes involved in the phenotype of mice with mutations in the EP300 KIX domain (EP300, CREBBP, CREB1, and MYB) (Figure 7). Messenger RNA 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 spleen of affected foals upon comparison to healthy foals. MYB mRNA expression appeared to be absent from FPS1 and FPS2 tissues, in contrast to the bone marrow and spleen 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 an 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 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 (Figure 8). PAX5 mRNA transcript numbers were low in the bone marrow of FPS1 and FPS2 foals (272 and 615 copies, respectively) in contrast to age-matched healthy Warmblood foals (16,967 and 3,030 copies). A similar pattern of PAX5 mRNA expression was observed in the spleen. The number of CREB1 transcripts detected in FPS1 and FPS2 was comparable to age-matched healthy Warmblood control foal tissues. Transcript numbers of MYB were low in FPS2 bone marrow only in contrast to FPS1 (2.1 x 10^8 versus 4.5 x 10^{10} copies) and healthy Warmblood control foal tissues (1.1 x 10^{10} and 2.3 x 10^{10} copies). 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 were identified in the coding sequence of either CREB1 or MYB compared to the healthy foal sequence or the GenBank predictions (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 in 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: a) FPS-affected foals may be born with erythroid precursors in the bone marrow and hematocrits and peripheral blood B cell distribution equivalent to that of healthy unaffected foals, suggesting a productive hematopoiesis during fetal life; b) perinatal development of a progressive B lymphopenia and anemia; c) bone marrow cytologic evidence of initial erythroid hyperplasia with dysplasia, followed by a rapid and progressive hypoplasia and aplasia, along with anemia; and d) 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 post-mortem samples, which was accompanied by low PAX5 gene expression in the bone marrow. Altogether, our data suggests 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 morphologic 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, an important question remains on the importance of 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. Altogether, the erythroid hypoplasia/dysplasia/aplasia, B cell lymphopenia/depletion, and myeloid dysplasia suggest a common underlying defect affecting the hematopoietic stem cell lineage differentiation versus 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 further elucidate the cell differentiation defect. Similar morphologic 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 - 8 days; therefore, maternally-derived IgM is depleted in the foal 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 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 post-suckling serum IgM concentrations close to birth were within the range observed for healthy Fell Pony foals, likely due to colostral transfer. Without analysis of pre-suckle
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 longer half-life of 23 - 30 days, thus the normal serum IgG concentrations measured in older affected foals at the time of death or euthanasia were likely due to 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 increase in total lymphocyte counts with age in 2 FPS-affected foals, and the absence of T cells in the bone marrow in 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 clearing the pathogen (27, 47). CD4+ and CD8+ 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 as 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 were expressed in FPS-affected and healthy foals, with exception of PAX5 and, potentially, MYB. PAX5 is essential for B lineage commitment and development, as well as 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 bone marrow and spleen of FPS-affected foals in contrast to 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 non-synonymous 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 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 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 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 in the rapid progression of the syndrome, observed failure in hematopoiesis, introduced the finding of myeloid dysplasia, and highlighted the importance of early testing before clinical signs are fully manifested. Most B cell differentiation essential genes, and genes associated with erythropoiesis are expressed in the affected foals, and a potential common hematopoietic genetic defect is still under investigation. Further, this primary immunodeficiency is a unique natural model to study 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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FIGURE LEGENDS

Figure 1. Sequential hematocrits of FPS-affected and unaffected Fell Pony foals from birth to 18 weeks of life.
At birth, FPS-affected foals (n = 2, filled shapes; data not available for FPS3) had hematocrits below or within the low range of the unaffected foals (n = 29, open diamonds), and all 3 FPS-affected foals became severely anemic by 4 weeks of life. Foals were sampled sequentially, although not all foals were sampled at each time point.

Figure 2. Sequential total lymphocyte counts of FPS-affected and unaffected Fell Pony foals from birth to 18 weeks of life.
At birth, FPS-affected foals (n = 3, filled shapes) had comparable total lymphocyte counts to age-matched healthy control foals (n = 29, open diamonds). Two of the affected foals failed to increase lymphocyte counts with age. One affected foal had comparable lymphocyte counts to unaffected foals at 6 weeks of life. Foals were sampled sequentially, although not all foals were sampled at each time point.

Figure 3. Sequential flow cytometric assessment of peripheral blood lymphocyte subpopulation distribution in FPS-affected and unaffected Fell Pony foals from birth up until 21 weeks of life, and adult Fell Pony horses.
Lymphocyte subpopulations were expressed as the percentage of positive cells for the selected marker within the defined lymphocyte gate in flow cytometric dot plot analyses. FPS-affected foals (n = 2, filled shapes; data not available for FPS1) were born with a
peripheral blood B cell subpopulation distribution (CD19-like+ IgM+) comparable to unaffected foals (n = 46, open diamonds) and adult Fell Pony horses (n = 6, open squares). 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 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 unaffected foals. Foals were sampled sequentially, although not all foals were sampled at each time point.

Figure 4. Sequential serum IgM concentrations in FPS-affected and unaffected Fell Pony foals from birth (post-suckle) to 18 weeks of life, and adult Fell Pony horses.

No increase in IgM concentrations with age was measured in post-suckle serum samples of FPS-affected foals (n = 3, filled shapes), in contrast to unaffected Fell Pony foals (n = 35, open diamonds). Results for 2 adult Fell Pony horses overlap. Foals were sampled sequentially, although not all foals were sampled at each time point.

Figure 5. 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 - a binucleate prorubricyte; D - a binucleate prorubricyte (arrow), abnormal
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Figure 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 control. No B cells were detected in the bone marrow and lymph nodes of FPS-affected foals. Splenic germinal centers revealed a paucity of B cells in FPS-affected foals, in contrast to the age-matched healthy foal spleen. The distribution of T cells in the lymph node and spleen is similar in all foals, however T cells were not present in the bone marrow of FPS1.

Figure 7. mRNA expression of selected B cell and erythrocyte developmental genes in the bone marrow and spleen of FPS-affected Fell Pony foals and age-matched healthy Warmblood foals.

The mRNA expression of most investigated genes is not different in the bone marrow of FPS-affected foals (FPS1, FPS2) compared to the healthy foal samples (H; n=2), except for CREB1, MYB, and PAX5, which showed decreased mRNA expression in both tissues. RT-PCR amplification of the \( \beta\)-actin (ACTB) gene was included as a cDNA quality control.
Figure 8. Quantitative RT-PCR of selected genes in the bone marrow and spleen of FPS-affected Fell Pony foals and healthy Warmblood foals.

The expression of PAX5 mRNA transcripts is decreased in both tissues from FPS-affected foals (FPS1, FPS2) compared to the healthy foals (H; n=2). Statistical comparison was not performed due to the small sample size. Bars represent the mean plus standard deviation of each sample performed in triplicate.
Table 1. Genes of interest sequenced from FPS-affected Fell Pony foals.

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<tr>
<th>Gene</th>
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<td>JN979559</td>
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¹Nucleotide sequence length excluding primers.
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