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Title Page

**Evidence of receptor-mediated elimination of erythropoietin by
analysis of Epo receptor mRNA expression in bone marrow and
erythropoietin clearance during anemia**

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Running title page

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- d) Nonstandard abbreviations:**
- | | |
|-------------------|-----------------------|
| Epo | erythropoietin |
| EpoR | Epo receptors |
| $\Delta\Delta$ CT | comparative CT method |
| rHuEpo | recombinant human Epo |
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Abstract

Erythropoietin (Epo) is the primary hormone that stimulates the erythroid proliferation and differentiation through its cell surface receptor (EpoR) on erythroid progenitor cells. Previous studies have suggested that the bone marrow plays an important role in Epo's elimination. The changes in the EpoR mRNA levels and Epo's clearance in the bone marrow of 11 newborn lambs were studied to elucidate the role of EpoR in Epo's clearance under anemic conditions. Epo mRNA levels were measured by real-time PCR and relative expression of EpoR was calculated using the comparative CT method ($\Delta\Delta CT$). The GAPDH house keeping gene was chosen as a control gene for the calculations. All lambs showed significant increase in bone marrow EpoR mRNA levels following phlebotomy-induced anemia. Epo's clearance determined from simultaneous pharmacokinetic studies with ^{125}I -rHuEpo showed a significant increase following phlebotomy-induced anemia that was similar to the increase in EpoR. By day 28 post phlebotomy, EpoR mRNA levels and Epo clearance had returned back toward baseline. These results indicate that the changes in Epo's clearance are not due to body growth but result from significant changes in the pool of EpoR. A linear mixed effect model was used to evaluate the quantitative relationship between EpoR and Epo's clearance. This analysis demonstrated a highly significant positive linear correlation between EpoR and Epo clearance. Together, these findings provide strong evidence that receptor mediated Epo clearance is an important route for Epo's elimination.

Introduction

Erythropoietin (Epo) is a glycoprotein hormone that stimulates the erythroid proliferation and differentiation (Jelkmann, 2007). Epo exerts its erythropoietic effects through a cell surface receptor (EpoR) on the erythroid progenitor cells (BFU-Es and CFU-Es) (Jelkmann, 2004; Richmond et al., 2005). Although EpoR expression has been reported in other cell types and tissues, the highest density of EpoRs are detected on erythroid progenitor cells in bone marrow (Rossert and Eckardt, 2005).

Binding of Epo to the cell surface induces dimerization of two EpoR molecules, which in turn initiates intracellular signal transduction required for the production of mature red blood cells (RBC) (Remy et al., 1999; Elliott et al., 2008). Human *EpoR* gene has been cloned from a placenta genomic library and characterized intensively (Maouche et al., 1991; Noguchi et al., 1991). Different EpoR isoforms derived by alternative splicing have been reported for the human, mouse, rat and ovine EpoR genes (Kuramochi et al., 1990; Nakamura et al., 1992; Yamaji et al., 1996; David et al., 2002).

Although the mechanisms involved in Epo elimination and site of degradation is still not completely understood, several studies suggest that receptor-mediated elimination of Epo plays an important central role in its clearance (Chapel et al., 2001a; Chapel et al., 2001b; Freise et al., 2007; Widness et al., 2007).

The hypothesis of an Epo elimination mechanism *via* non-recycled EpoR receptors is supported by several observations. Firstly, Epo's clearance shows transient perturbations in conjunction with large transient perturbations in endogenous Epo levels (Chapel et al., 2001b; Freise et al., 2007; Widness et al., 2007). Secondly, the Epo clearance is significantly reduced following busulfan-induced bone marrow ablation (Chapel et al., 2001a). Although these observations are compelling, they only provide indirect evidence for Epo elimination being primarily EpoR-mediated. Thus, the current study was undertaken to

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provide additional direct evidence of the proposed EpoR-based elimination mechanisms by analysis investigating the relationship between the EpoR mRNA levels and Epo clearance. Experimentally this was done by investigating the changes in Epo clearance in association with serial, simultaneously measured EpoR mRNA levels in the bone marrow following a phlebotomy-induced anemia in lambs.

Methods

Animals and study outline:

All animal experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee prior to the study. Eleven lambs, 3-4 weeks old, were studied. The lambs were housed in an indoor, light- and temperature-controlled environment alongside of their mothers. Jugular venous catheters used for blood sampling were inserted under pentobarbital anesthesia. Ampicillin (1g/day) was administered for the first 3 days following the catheter insertion. Phlebotomy-induced anemia was achieved by removing 60% of the estimated total body RBC volume from the lambs starting from initial basal Hb levels of 9.7 ± 1.1 g/dL (mean \pm standard deviation). This was accomplished by exchange phlebotomy where equal volumes of autologous plasma (or saline when the plasma volume was insufficient) were transfused for each volume of blood removed. Doing so decreased the lamb's Hb level to 3.7-4.2 g/dL. For all 11 lambs a baseline Epo PK study and bone marrow aspiration procedure were performed the day before the major phlebotomy and repeated again at approximately 9 days following phlebotomy. In 6 of the 11 lambs an additional Epo PK study and bone marrow aspiration were performed approximately 28 days after the major phlebotomy (Fig.1) when the Hb levels reached were not statistically different from the basal levels ($p < 0.05$)(9.5 ± 1.5 g/dL). Throughout the study, whole blood samples

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were analyzed using a XT 2000 automated hematology system analyzer (Sysmex America Inc. Mundelien, IL).

PK studies:

Following intravenous bolus dose of a tracer amount of ^{125}I -rHuEpo (less than 0.1 U rHuEpo/kg), 14 blood samples were taken over the subsequent 7-hour period. Plasma concentration of ^{125}I -rHuEpo were analyzed by a double antibody immunoprecipitation assay as previously described (Widness et al., 1992) with a lower level of detection of 0.004 mU/ml and a coefficient of variation of 7 percent in the accuracy.

Bone marrow aspirate and peripheral blood collection and RNA isolation

Bone marrow aspirates (2 ml) were collected from the iliac crest into a 5 ml syringe containing heparin (1000 units/ml) using a sterile 18 gauge bone marrow aspiration needle. Bone marrow aspirates were transferred into 2 ml centrifuge tubes and spun at 1000g for 5 minutes after which the plasma was removed and the cell pellet was mixed with RNAlater Solution (RiboPureTM-Blood Kit, Ambion) and stored at -20°C. Total RNA was isolated from stored bone marrow samples using the RiboPureTM-Blood Kit from Ambion, Inc. Samples were treated with amplification grade DNaseI (Invitrogen) to ensure the DNA free RNA. Peripheral blood samples (2 ml) were obtained simultaneously as marrow samples and RNA was isolated in a similar matter.

Reverse Transcription and Real-time PCR:

Complementary DNA (cDNA) was generated from 0.5 µg of total RNA using Superscript III Reverse transcriptase (Invitrogen) according to manufacturer's protocol. Real-time PCR reactions were performed in triplicate in 384-well plates with an ABI Prism 7700 Sequence

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Detector (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied BioSystems). Minus RT reactions were also run each time to test for genomic DNA contamination. Real-time PCR primers for sheep EpoR and GAPDH (house keeping gene) genes were designed using partial Ovis Aries cDNA sequences (AY029231 and U94889, respectively). All primers were designed either to span exon-exon junctions or to anneal to different exons thereby preventing genomic DNA amplification. The comparative CT method ($\Delta\Delta CT$) was used to calculate relative expression of EpoR. The amplification efficiencies of EpoR primer sets (EpoRI, EpoRII and EpoRIII) and GAPDH primer set were confirmed to be approximately equal before the study samples were analyzed.

The primer sequences used were: ovGAPDH F: 5'ACAGCGACTCACTCTTCTACCT; R: 5'CCC TGT TGC TGT AGC CGA ATT CAT; ovEpoR I F: 5'TCG TGC TCA TTC TAC TGC TGC T, R: 5'ATG CCA GGC CAG ATC TTC TGC TT; ovEpoR II F: 5'ACA AGG GTA ACT TCC AGC TGT GG, R: 5'AGC ACT CAG AGA GGA CTT CCA AGG; ovEpoR III F: 5'CTC CTG TGG CCG AAC TCC AAG TCT, R: 5'AGA CAG CCG TCA GTC TGG TAC A

Pharmacokinetic analysis:

The Epo plasma concentration profile from a single intravenous bolus of ^{125}I -rHuEpo was best described by a bi-exponential distribution function. Curve fitting was performed using WINFUNFIT, a Windows (Microsoft) version evolved from the general non-linear regression program FUNFIT (Pedersen, 1977). To account for weight change, Epo clearance values throughout this article are clearance values normalized to the bodyweight.

Statistical analysis:

The statistical analysis was conducted using R software (Version 2.6.1; <http://cran.r-project.org/>). Repeated measure ANOVA was conducted to assess the effect of phlebotomy on mRNA levels and clearance values. Since our data include both fixed and random effects, repeated measure ANOVA was applied using lme (linear mixed effect) function in R

(nlme library). Tukey's test for multiple comparisons were applied using glht (general linear hypothesis testing) function in R (multcomp library). The following linear mixed model was used to examine the relationship between Epo clearance values and EpoR mRNA levels:

$$Cl_{i,j} = \alpha_i + \beta_i \cdot mRNA_{i,j} + \varepsilon_{i,j} \quad (1)$$

$$\varepsilon_{i,j} \sim N(0, \sigma_\varepsilon^2) \quad (2)$$

$$\begin{pmatrix} \alpha_i \\ \beta_i \end{pmatrix} \sim BVN \left(\begin{pmatrix} \alpha \\ \beta \end{pmatrix}, \begin{pmatrix} \sigma_\alpha^2 & 0 \\ 0 & \sigma_\beta^2 \end{pmatrix} \right) \quad (3)$$

where, $Cl_{i,j}$ is the j^{th} Epo clearance for the i^{th} subject. The $mRNA_{i,j}$ term denotes the j^{th} EpoR mRNA relative value for the i^{th} subject; α_i and β_i are the slope and the intercept for the i^{th} subject; $\varepsilon_{i,j}$ is residual error, and σ^2 is the variance of the bivariate normal distribution, BVN.

Normal distribution was used to express interindividual variability with diagonal variance covariance matrix (Eqs.1-3). An additive model was used to describe the residual error.

Since absolute number of EpoR mRNA molecules cannot be ascertained by real time PCR, the relative number of EpoR mRNA molecules was derived from cycle number of gene of interest, EpoR, and house keeping gene, GAPDH, as shown below. The relative number of EpoR mRNA molecules does not indicate the exact number of genes, instead it gives a value proportional to the actual number (Pfaffl, 2001). While this proportionality constant cannot be determined with the current analysis, it was the same for all samples analyzed.

This assumes that the efficiency of the target gene is 2.

$$mRNA_{i,j} = 2^{-\Delta CT(\text{Sample})} \quad (4)$$

and

$$\Delta CT = CT_{EpoR} - CT_{GAPDH} \quad (5)$$

where, CT_{EpoR} is the cycle number of EpoR mRNA, and CT_{GAPDH} is the cycle number of GAPDH.

Results

The partial ovine EpoR cDNA sequences for sheep, spanning from exon 2 to exon 8, has been published (David et al., 2002). The PCR primers designed to amplify ovine EpoR amplified the two distinct expected bands (Fig.2A). A 93bp insert between exons 7 and 8 potentially results in expression of a longer EpoR protein as described earlier (David et al., 2002). We designed 3 different primer sets (EpoRI, EpoRII and EpoRIII) (Fig.2B) to amplify the different isoforms independently. EpoRI primer set amplifies both isoforms whereas EpoRII and EpoRIII primer sets specifically amplify longer and shorter isoforms of the EpoR cDNA respectively. Total RNA isolated from bone marrow (BM) and peripheral blood (PB) samples were used to generate cDNA. Cell surface EpoR protein expression is found on BFU-Es and CFU-Es located within the bone marrow space, and is not found on mature RBC or other cellular components of peripheral blood (Broudy et.al. 1991) Thus, peripheral blood samples served as negative controls for EpoR mRNA expression profiles. As expected, EpoR I, EpoRII and EpoRIII amplified EpoR cDNA only in BM samples (Fig.2C). The internal housekeeping gene, GAPDH, was equally expressed in BM and PB (Fig.2C, last panel). Experiments were undertaken to determine if different isoforms had different expression levels, and if isoform expression levels are affected by phlebotomy induced anemia. PCR analysis revealed that under steady-state baseline (non-anemic) conditions that the shorter isoform tends to be more abundant than the longer EpoR mRNA isoform (Fig.2A). In addition, the levels of these two isoforms were affected in a very similar manner under conditions of phlebotomy induced anemia. Therefore we have used real-time PCR results obtained from EpoRI primers for the statistical analyses.

Real time-PCR analyses revealed that 9 days after the major phlebotomy EpoR mRNA levels were higher than pre-phlebotomy baseline levels in all study lambs. The increases for the EpoR mRNA levels ranged between 1.2 and 12.6-fold (Table 1). The six lambs that had

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an additional bone marrow aspirate collected at day 28 post-phlebotomy had Epo mRNA levels that were all significantly lower than those measured at day 9.

As shown in Table 2, repeated measure ANOVA analysis of EpoR mRNA levels revealed that compared to baseline day -1, there was a significant ($p < 0.05$) increase in EpoR mRNA levels by 9 days post-phlebotomy, which subsequently decreased in all 6 lambs by 28 days post-phlebotomy. There was no significant difference between EpoR mRNA levels before phlebotomy and 28 days post-phlebotomy.

These sequential changes observed in EpoR mRNA over the four-week study period paralleled those observed for Epo clearance. Ten out of 11 subjects showed an increase in Epo clearance on day 9 post-phlebotomy, decreasing toward baseline levels by day 28. The repeated measure ANOVA analysis conducted to examine the effect of phlebotomy on Epo's clearance demonstrated that there is a significant increase in Epo's clearance 9 days post-phlebotomy compared to both the Epo clearance before phlebotomy and 28 days post-phlebotomy ($p < 0.05$). Similar to what was observed for EpoR mRNA levels, Epo's clearance before the phlebotomy was not significantly different from that at 28 days post-phlebotomy (Table 2).

The linear mixed effect modeling of Epo's clearance using mRNA as a covariate showed that the clearance is positively and linearly related to mRNA by a population slope of 76.8 and a population intercept of 63.1. The 95% confidence interval of the population estimate for the slope is 13.7-139 and for the intercept is 51.1-75.1. Regarding interindividual variability, the coefficient of variation as percent for the individual estimates for the slope is 125.8 and for the intercept 51.3. The clearance values estimated by the linear mixed effect model plotted against the actual measured clearance are shown in Fig.3.

Discussion

The present study provides compelling new additional evidence that EpoR mRNA is up-regulated as a result of phlebotomy-induced anemia. In further support of this hypothesis is the report that the incubation of murine cell lines with Epo for 6 days increases the number of EpoR per cell without changing EpoR binding affinity (Broudy et al., 1990). EpoR up-regulation was also reported in rat brain endothelial cell line and cortical astrocyte cell cultures as a result of hypoxic conditions (Bernaudin et al., 1999). Our observation that EpoR mRNA levels decreased towards baseline 28 days after phlebotomy relative to the 9-day sample supports our hypothesis that EpoR up-regulation is due to increased endogenous Epo levels following phlebotomy and is not a developmental growth phenomena.

Since anti-ovine EPOR antibody is not commercially available it was not possible to show the changes in EPOR protein levels. Spandou et al. 2004 reported that after hypoxia-ischemia up regulation of EPOR mRNA and protein levels show very similar temporal pattern in rat brain. Therefore, we expect the EPOR protein levels to show similar pattern to EPOR mRNA levels.

Our previous studies provided significant evidence that bone marrow plays a major role in Epo elimination (Chapel et al., 2001a; Widness et al., 2007) and that EpoR is upregulated as a result of phlebotomy-induced anemia in sheep (Freise et al., 2007). Similarly, the present study showed an increase in Epo clearance 9 days after phlebotomy. As was observed with EpoR mRNA, the increase observed in Epo clearance is not likely a natural developmental event resulting from growth. This speculation is supported by the observation that Epo clearance decreased toward baseline by 28 days post-phlebotomy. Taken

together, these findings strongly support the hypothesis that Epo elimination is largely the result of a receptor-mediated mechanism.

There was a close similarity observed in the behavior of changes in EpoR mRNA and Epo clearance throughout the study during the transition from baseline conditions followed by anemic stress conditions as a result of phlebotomy with a subsequent return towards baseline. This observation supports our previously published receptor-mediated model of Epo elimination (Chapel et al., 2001b). Furthermore, results from our linear mixed effect model show a linear relationship between EpoR mRNA and Epo clearance. The positive slope observed is significantly different from zero. This indicates that Epo's clearance increases linearly in parallel with the amount of EpoR mRNA in erythroid progenitor cells. The positive intercept can be explained by a simultaneous Epo elimination through a non-hematopoietic elimination pathway. Studies by other research groups also provide a substantial indirect evidence of receptor-mediated elimination of Epo. Sawyer et al. demonstrated receptor-mediated elimination of Epo as a result of lysosomal degradation in erythroid cells (Sawyer et al., 1987). In rats, higher organ specific uptake of ^{125}I -rHuEpo in the bone marrow was also observed (Spivak and Hogans, 1989). Gross and Lodish also reported that 40% of Epo internalized in cells carrying EpoR is intracellularly degraded (Gross and Lodish, 2006).

EpoR mRNA splicing variants have been reported for human, mouse, rat and sheep previously (Kuramochi et al., 1990; Nakamura et al., 1992; Yamaji et al., 1996; David et al., 2002). However, the role of different EpoR transcripts has not been well understood. In humans the splice variant with 95 bp insert between exons 6 and 7 introduces an early stop codon generating a truncated receptor (Nakamura et al., 1992). However, the sheep EpoR transcript variant potentially encodes a longer protein in addition to wild type EpoR, since the 93 bp insert does not interrupt the open reading frame (David et al., 2002).

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We investigated whether phlebotomy induced anemia has separate effects on expression of EpoR mRNA isoforms. We observed that the two EpoR mRNA isoforms were expressed significantly higher following phlebotomy, with no significant change in the ratio of the two isoform transcripts. Further studies are required to investigate whether the longer EpoR isoform is translated in to a functional protein and has different characteristics than wild type EpoR.

While it is difficult to predict whether the current findings extrapolate to EpoR expressing-non-hematopoietic cells, we propose that Epo's actions are mainly on the hematopoietic system, Epo has been shown to exert cardioprotective and neuroprotective effects (Calvillo et al., 2003; Jelkmann, 2007). As mentioned above, EpoR up-regulation was also reported in rat brain endothelial cell line and cortical astrocyte cell cultures as a result of hypoxic conditions (Bernaudin et al., 1999). Thus one would expect a similar regulatory effect on Epo's neuroprotective properties.

Linear mixed model was used because it is suitable for dealing with sparse sampling and unbalanced design as in the current study. The data analyzed by the mixed model appeared challenging and several programs were tried including NONMEM and SAS both of which did not convergence successfully. Fortunately, successful convergence was obtained using the lme function available in R, but the relative standard error for interindividual estimates could not be determined using R.

In summary, based on the analysis of data from this study and previous findings in the literature we suggest that under conditions of severe tissue hypoxemia, such as occurs with anemia severe enough to result in a high degree of Epo stimulation, hematopoietic EpoR are up-regulated as a part of a feedback regulation. The feedback regulation is triggered by a decrease in tissue oxygen (pO_2). The pO_2 in turn depends on different physiological variables including hemoglobin concentration, arterial pO_2 , hemoglobin affinity to oxygen,

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and rate of blood flow. The decrease in pO_2 induces an increase in the endogenous Epo production. Increases in Epo stimulate erythropoiesis by several mechanisms that includes preventing the apoptosis of colony forming unit erythroid (CFU-E) (Koury and Bondurant, 1990). Each CFU-E will produce 8 to 60 erythroblasts (Jelkmann, 2007). We also propose that Epo stimulates erythropoiesis by up-regulating EpoR number per cell. Increasing EpoR number will result in an increase in the efficacy of Epo in stimulating erythropoiesis.

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Footnotes

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Legends for Figures

Figure 1. Graphical representation of the study outline. BM: Bone marrow aspirate collection; PK: Pharmacokinetic assay; n: sample numbers.

Figure 2. Two isoforms of EpoR and EpoR real-time PCR primer locations.

A. Ethidium bromide stained agarose gel. The two isoforms of EpoR are indicated with arrows. **B.** Exons are shown as gray solid boxes and 93 bp insertion as crossed-line pattern box. Arrows indicate real time PCR locations. **C.** Ethidium bromide stained agarose gels for each EpoR real time primer pair and GAPDH. M: DNA bp marker; BM: Bone marrow; PB; Peripheral blood.

Figure 3. Estimated clearance values using linear mixed effect model vs. actual clearance values (ml/hr/kg)

Tables

Table 1. Fold Changes in EpoR mRNA levels and Epo Clearance Pre- and Post-Phlebotomy

Lamb ID	Fold change in EpoR mRNA levels relative to pre-phlebotomy			Fold change in Epo Clearance relative to pre-phlebotomy		
	Pre-phlebotomy	9 d post-phlebotomy	28 d post-phlebotomy	Pre-phlebotomy	9 d post-phlebotomy	28 d post-phlebotomy
1	1.0	12.6	ND	1.0	2.3	ND
2	1.0	5.0	ND	1.0	1.3	ND
3	1.0	9.5	ND	1.0	1.2	ND
4	1.0	2.8	ND	1.0	1.4	ND
5	1.0	3.7	ND	1.0	1.8	ND
6	1.0	3.5	2.3	1.0	2.2	1.4
7	1.0	1.2	0.5	1.0	1.5	0.8
8	1.0	2.4	1.1	1.0	1.4	0.8
9	1.0	2.2	1.6	1.0	1.3	0.6
10	1.0	10.2	3.0	1.0	0.9	0.6
11	1.0	1.6	0.8	1.0	1.8	1.0

N.D.: Not Done

Table 2: Repeated measure ANOVA results for EpoR mRNA levels and Epo linear clearance.*

	Estimated difference	Standard error	P value
$Cl_L^2 - Cl_L^1$	32.7	6.16	<0.0001**
$Cl_L^3 - Cl_L^1$	-9.3	7.41	0.42
$Cl_L^3 - Cl_L^2$	-42.0	7.41	0.0001**
mRNA ² - mRNA ¹	0.16	0.03	0.0001**
mRNA ³ - mRNA ¹	0.04	0.04	0.58
mRNA ³ - mRNA ²	-0.12	0.04	0.01**

* Cl_L^1 , Cl_L^2 , and Cl_L^3 are clearance (ml/hr/kg) pre-phlebotomy, 9 days post-phlebotomy and 28 days post-phlebotomy respectively. mRNA¹, mRNA², and mRNA³ are mRNA values pre-phlebotomy, 9 days post-phlebotomy, and 28 days post-phlebotomy.

** Indicate significant difference from zero at level of significance < 0.05

Figure 1

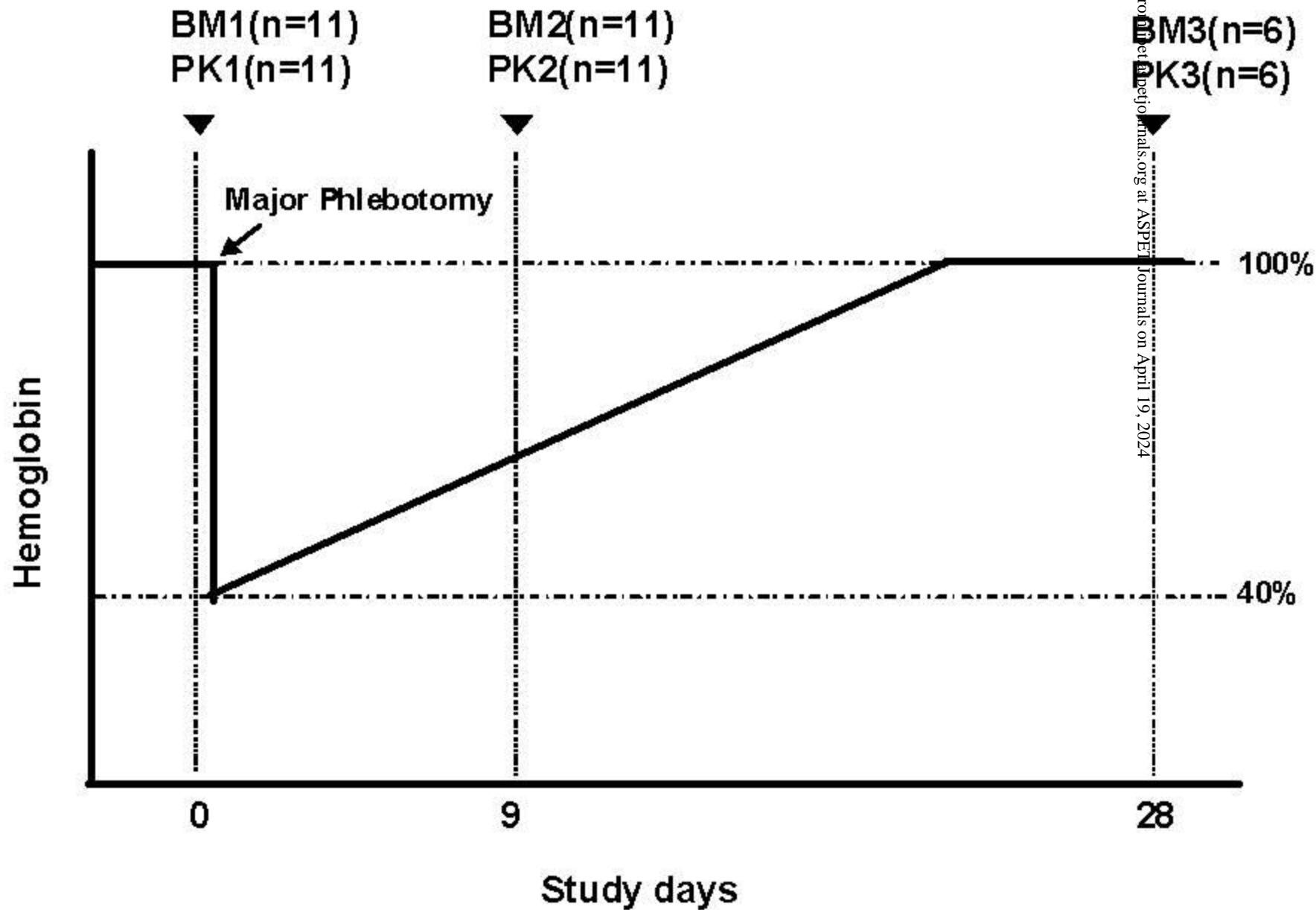


Figure 2

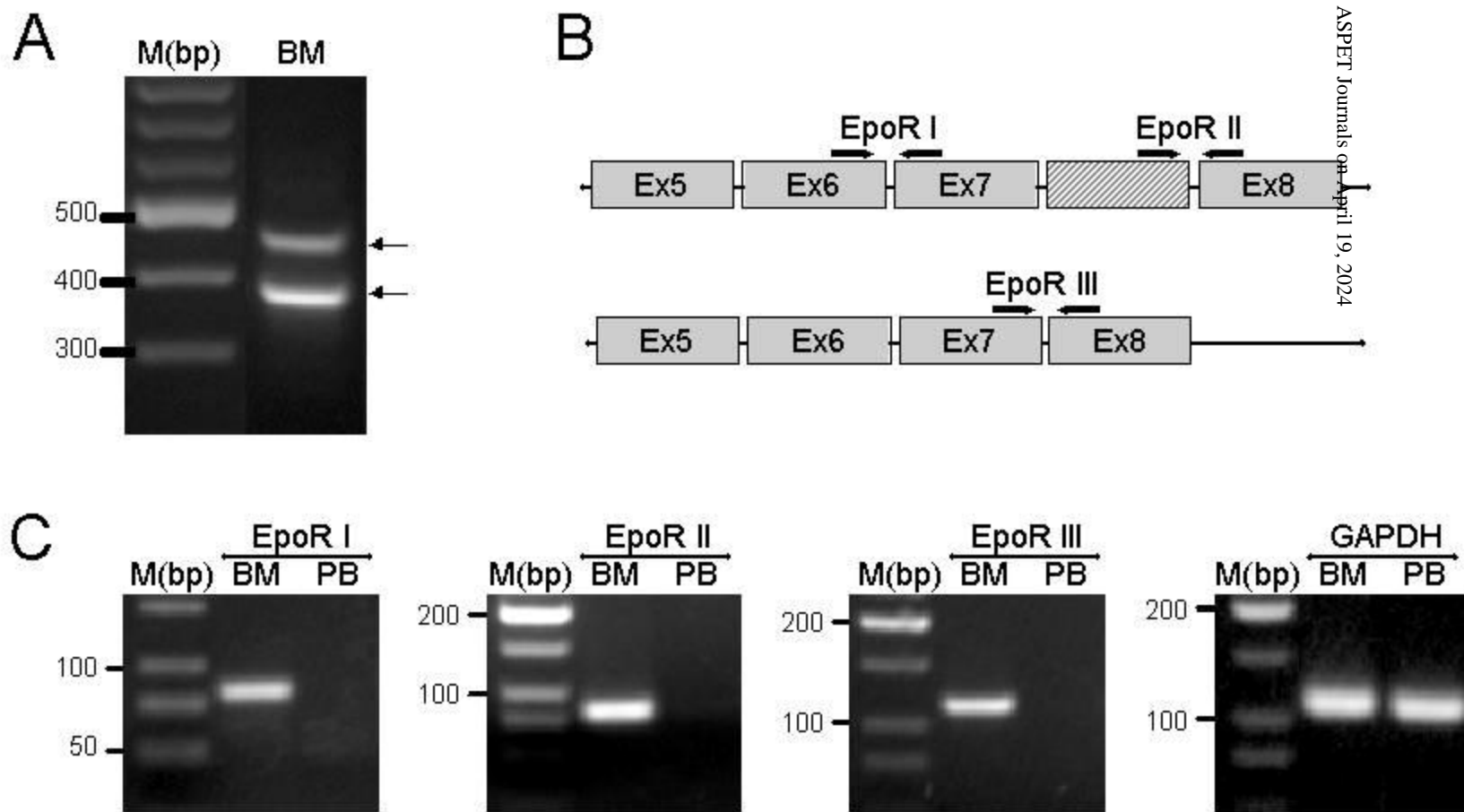


Figure 3

