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**Alterations in Methylation of the Erythropoietin Gene During Human Fetal  
Development**

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## **Abstract**

**Background:** Both hypoxia and anemia stimulate erythropoiesis by stimulating Epo transcription and protein production. Developmental, tissue-specific, and environmental signals all contribute to the precise regulation of the Epo gene. Temporal and tissue-specific signals limit expression of the Epo gene primarily to cells in the fetal liver and adult kidney. The precise mechanisms regulating Epo gene expression during human fetal development are unclear. We sought to determine if regulation of the Epo gene occurs in some part through methylation. Using the demethylating agent 5-aza-2' deoxycytidine (DAC), we compared Epo mRNA expression in human fetal kidney and liver between 12 and 22 weeks gestation under normoxic and hypoxic conditions.

**Methods:** Primary cell cultures from liver and kidney tissue ranging from 12-22 weeks were either treated with DAC or vehicle control for three days. After day three, each matched kidney and liver set were incubated at 1% (hypoxia) or 21% (room air) for eight hours in a 37 degree C incubator. RNA was harvested using TriZol, isolated, reverse transcribed, and quantification PCR was performed to measure Epo mRNA expression. Epo mRNA expression was normalized to an internal standard 18S rRNA in each duplex reaction.

**Results:** Epo mRNA concentrations were much greater in liver than kidney at all gestations tested ( $p < 0.001$ ). A twenty-fold increase in Epo mRNA concentrations occurred when liver samples were exposed to hypoxia, however this increase was not enhanced by pretreating the samples with DAC. There was no statistical difference in Epo mRNA concentrations when kidney samples were exposed to hypoxia. There was a four-fold increase in Epo mRNA concentrations when kidney samples were pretreated with DAC, however this difference was not statistically significant.

**Conclusions:** Demethylation of fetal kidney increased Epo mRNA expression, but not to the level of Epo mRNA expression measured in fetal liver. Methylation of fetal liver did not increase Epo mRNA expression under hypoxic conditions. We speculate that Epo gene expression in fetal kidney is regulated in part by methylation, and is developmentally regulated during mid-gestation.

**Abbreviations:** Epo (erythropoietin), DAC (5-aza-2'-deoxycytidine), DMEM (Dulbecco's Modified Eagle's Medium)

## **Introduction**

Regulation of the red blood cell mass relies on modulating erythropoietin (Epo) gene expression in response to tissue oxygen tension. Both hypoxia and anemia stimulate erythropoiesis by stimulating Epo transcription and protein production. Developmental, tissue-specific, and environmental signals limit expression of the Epo gene primarily to cells in the fetal liver. Epo gene expression transitions to the kidney only after birth. The precise mechanisms regulating Epo gene expression during human fetal development are unclear.

The regulation of gene expression can occur through biochemical, non-genetic alterations in the gene. Such mechanisms can occur through methylation – the process in which methyl groups attach to cytosines of CpG sites, thereby blocking gene expression through tertiary changes. Regulation via methylation has been proposed as a cause for tissue and developmental differences in Epo gene expression.

The purpose of this study was to determine if methylation played a role in Epo gene expression during fetal development. Regulation of the Epo gene occurred in part through methylation by exposing fetal tissues to a demethylating agent, 5-aza-2'-deoxycytidine (DAC). We sought to determine if methylation could affect Epo expression. We hypothesized that the Epo gene would be primarily unmethylated in fetal liver and would not respond to a demethylating agent by increasing Epo mRNA expression. We also hypothesized that fetal kidney would be heavily methylated and would therefore respond by increasing Epo mRNA expression. We compared Epo mRNA expression in human fetal

kidney and liver between 12 and 22 weeks gestation after pretreating cells with DAC and exposing to hypoxia.

## **Methods**

### *Tissue samples:*

Fetal liver and kidney samples were obtained 15 minutes after termination from six fetuses ranging 12 to 22 weeks gestation. After identifying liver and kidney tissue, the tissue was minced using sterile scalpel blades and placed in 10 mL of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. Tissue was triturated to create a cell suspension. Trypan blue exclusion was performed to determine cell viability. Viable cells were plated at a density of  $10^6$  cells per mL in 60 cm<sup>3</sup> culture dishes. Cells were placed in a 37°C incubator with 5% CO<sub>2</sub> and 21% O<sub>2</sub> and allowed to adhere to the plate for 24 hours.

Hep 3B cells constitutively express Epo at low levels under normoxic conditions, and express abundant Epo mRNA and protein in response to hypoxia. These cells served as positive controls. Previous studies showed that the promoter and enhancer regions of the Epo gene are primarily unmethylated. HeLa cells do not constitutively produce Epo under normoxic or hypoxic conditions and therefore served as negative controls.

### *Cell culture and processing:*

DAC (Sigma-Aldrich) was reconstituted in sterile water and diluted to a 10 mM working stock. Based on preliminary dose-response experiments (data not shown), a final concentration of 50 μM was chosen. After 24 hours, media was removed, cells were washed

with phosphate buffered saline (PBS) and, and fresh media containing DAC or vehicle control (sterile water) was added. Media was changed every 24 hours for three consecutive days.

After three days of DAC treatment, fetal cells, Hep 3B cells, and HeLa cells were incubated at 1% or 21% oxygen for eight hours. The cells were then removed from the incubator for processing. Total RNA was harvested using 1 mL of TriZol (Invitrogen, Carlsbad, CA).

RNA was isolated and quantified using a spectrophotometer. Reverse transcription was performed on 200ng total RNA using a commercially available kit (cDNA Archive Kit, Applied Biosystems, Foster City, CA).

#### *Quantitative-PCR:*

Quantification of the Epo mRNA was performed using the ABI Prism 7700 Sequence Detection System (ABI). PCR reaction volumes contained 4  $\mu$ L cDNA, 1  $\mu$ L Epo primer /probe mix (6-FAM-tgg aag agg atg gag gtc ggg ca- TAMRA) , 5' primer (5'-aat atc acg acg ggc tgt g-3') and 3' primer (3'-tgc cag act tct act gcc-5'), 10  $\mu$ L TaqMan Universal Master Mix, 1  $\mu$ L 18 S primer /probe mix (sequence copyright of ABI), and 4  $\mu$ L PCR water for a total reaction volume of 20  $\mu$ L.

Absolute quantification assay was performed using Hep 3B total RNA that was serially diluted (500ng, 100ng, 20ng, 4ng) prior reverse transcription to provide a standard curve of

Epo mRNA expression. Epo mRNA concentrations were normalized to 18S rRNA concentrations. Each duplex reaction (Epo and 18S) was run in duplicate.

Determination of quantity was based on the standard curve generated. The log quantity of cDNA standard was plotted against the cycle threshold, slope (m) any y-intercept (b). The cycle threshold of the sample was determined using the formula  $x \text{ (quantity)} = 10^{(Ct-b)/m}$ .

#### *Statistical Analysis:*

Epo expression for all samples was normalized to the internal 18S rRNA control expression based on the 18S mRNA concentration of 100 ng. The equation  $(100 \text{ ng } 18S / \text{measured } 18S \text{ concentration}) \times (\text{measured Epo concentration})$  allowed for this normalization. Epo mRNA concentrations were compared using unpaired t-tests and ANOVA (analysis of variance).

Differences in Epo mRNA concentrations resulting from the various experimental conditions ( $\pm$  DAC,  $\pm$  hypoxia, liver versus kidney) were analyzed using STATView commercial software (SAS institute, Version 5.01, Cary, NC). A power analysis was originally performed to determine the number of fetal samples to be studied, however due to time constraints only the first 6 samples are reported. The study was deemed not to constitute human subject research by the Human Research Review Committee at the University of New Mexico, as no identifiable patient data was collected.

## **Results**

Epo mRNA concentrations were much greater in liver than kidney at all gestations tested ( $p < 0.001$ ). A twenty-fold increase in Epo mRNA concentrations occurred when liver



samples were exposed to hypoxia ( $p=0.001$ ), however this increase was not enhanced by pretreating the samples with DAC ( $p=0.096$ ; figure 1). There was no statistical difference in Epo mRNA concentrations when kidney samples were exposed to hypoxia ( $p=0.168$ , figure 2). There was a four-fold increase in Epo mRNA concentrations when kidney samples were pretreated with DAC, however this difference was not statistically significant. When kidney samples were grouped into early (12-16 weeks) and late (17-22 weeks) gestation, there was a significant increase in mRNA concentrations in the early kidney samples pretreated with DAC and exposed to hypoxia ( $p=0.024$ ).

HeLa cells were similar to kidney samples, in that they responded to pretreatment with DAC by increasing Epo mRNA concentrations under hypoxic conditions ( $p=0.006$ , figure 3). Epo mRNA concentrations in Hep 3B cells pretreated with DAC were lower under both normoxic and hypoxic conditions ( $p=0.004$  and  $p<0.001$ , respectively, figure 4), however the response to hypoxia appeared preserved in that pretreatment with either DAC or vehicle control resulted in increased Epo mRNA concentrations in response to hypoxia ( $p=0.0005$ , DAC pretreatment;  $p=0.0002$ , vehicle control).

## Discussion

We hypothesized that demethylation of the enhancer region of the Epo gene with DAC would significantly increase mRNA expression in fetal kidney.

In these sets of experiments expression of Epo mRNA was significantly greater in fetal liver than fetal kidney. The greatest Epo expression occurred in Hep 3B cells not exposed to DAC and exposed to hypoxia. This is likely due to the fact that the enhancer region is unmethylated and therefore, would not increase Epo expression in response to pretreatment with a DNA methyltransferase inhibitor like DAC. In previous experiments, Epo expression was inhibited by DAC pretreatment (data not shown).

While Epo mRNA expression in fetal kidney cells of all gestational ages was markedly less abundant than fetal liver, treatment with DAC and exposure to hypoxia showed a seven-fold increase in Epo expression compared with kidney cells not treated with DAC and incubated in a normoxic environment. These findings help support our theory that regulation of the Epo gene occurs in part through methylation. Also, the cervical cancer cell line, HeLa, does not produce Epo and served as our negative control. When exposed to DAC and hypoxia, however, Epo expression increased four-fold as compared to the non-DAC treated, normoxic counterpart.

Epo production in the fetus occurs in the liver. Sometime after birth Epo production shifts to the kidney (1-3). The shift may be due to the changes in arterial oxygen tension occurring just after birth (4). Interestingly, Epo production drops dramatically within 24 hours of delivery, and both term and preterm infants experience a gradual decrease in hemoglobin concentration over the next 2 to 3

months. In term infants, Epo production and erythropoiesis resumes between 6 to 12 weeks of life. In preterm infants, the reinitiation of Epo production is delayed, and erythropoiesis does not occur. The resulting “anemia of prematurity” affects preterm infants less than 32 weeks gestation. It is characterized by a normochromic, normocytic anemia with decreased hemoglobin and reticulocytes. The decrease in hemoglobin in preterm infants is much more significant than that seen in term infants (5-8). Infants demonstrating symptoms of anemia are treated by transfusion (9, 10). The molecular and cellular mechanisms responsible for the anemia of prematurity are still undefined.

Erythropoietin (Epo) is a 34 kilodalton glycoprotein hormone located on chromosome 7q22 that stimulates the differentiation of lineage-committed pluripotent stem cells to erythrocytes in response to hypoxic conditions. Regulation of erythropoiesis and the red blood cell mass relies on altering Epo gene expression in response to tissue oxygen tension. Epo gene regulation in the adult requires an oxygen-sensing mechanism (11, 12). Both hypoxia and anemia stimulate transcription of the Epo gene in the kidney (13).

Studies evaluating cell lines such as Hep 3B cells that produce significant Epo protein in response to hypoxia reveal that binding sites for hypoxia response elements such as hypoxia inducible factor 1 (HIF-1) in the promoter and enhancer regions are free of methylation. Thus, 10 to 1000 fold enhancement of transcription of the Epo gene occurs. The human Epo gene contains critical CpG sites in the HIF-1 binding site of the enhancer region that may be differentially methylated.

DAC inhibits DNA methyltransferase in genomic DNA and causes demethylation of cytosine residues in CpG islands. Removal of bulky methyl groups allows transcription factors to bind to the promoter regions allowing assembly of transcription complexes and thus, gene expression.

Significant heterogeneity occurs when preparing primary cell cultures from fetal liver and kidney. These cell suspensions include Epo producing and non-Epo producing cells. Further studies in the laboratory will include ways to differentiate cells of the fetal liver and kidney that express Epo.

We speculate that methylation patterns are developmentally controlled and may vary with gestational age.

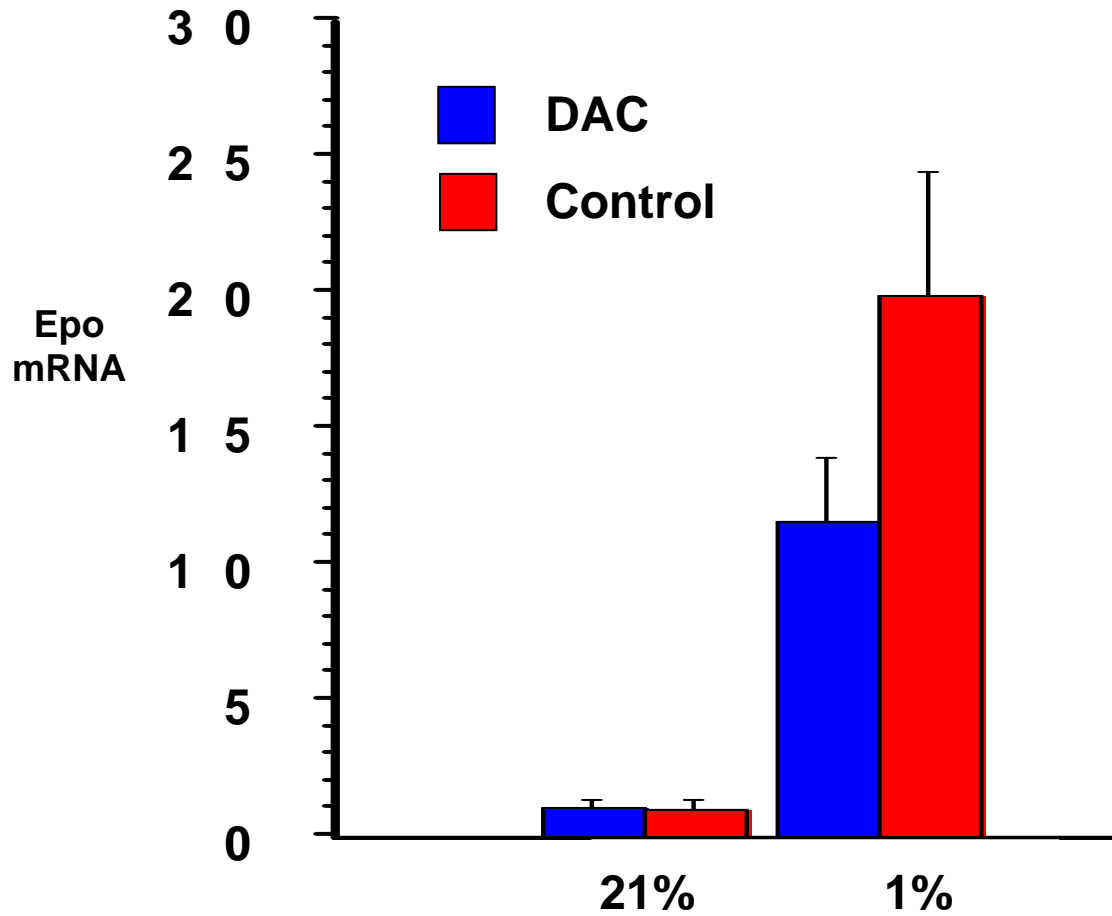
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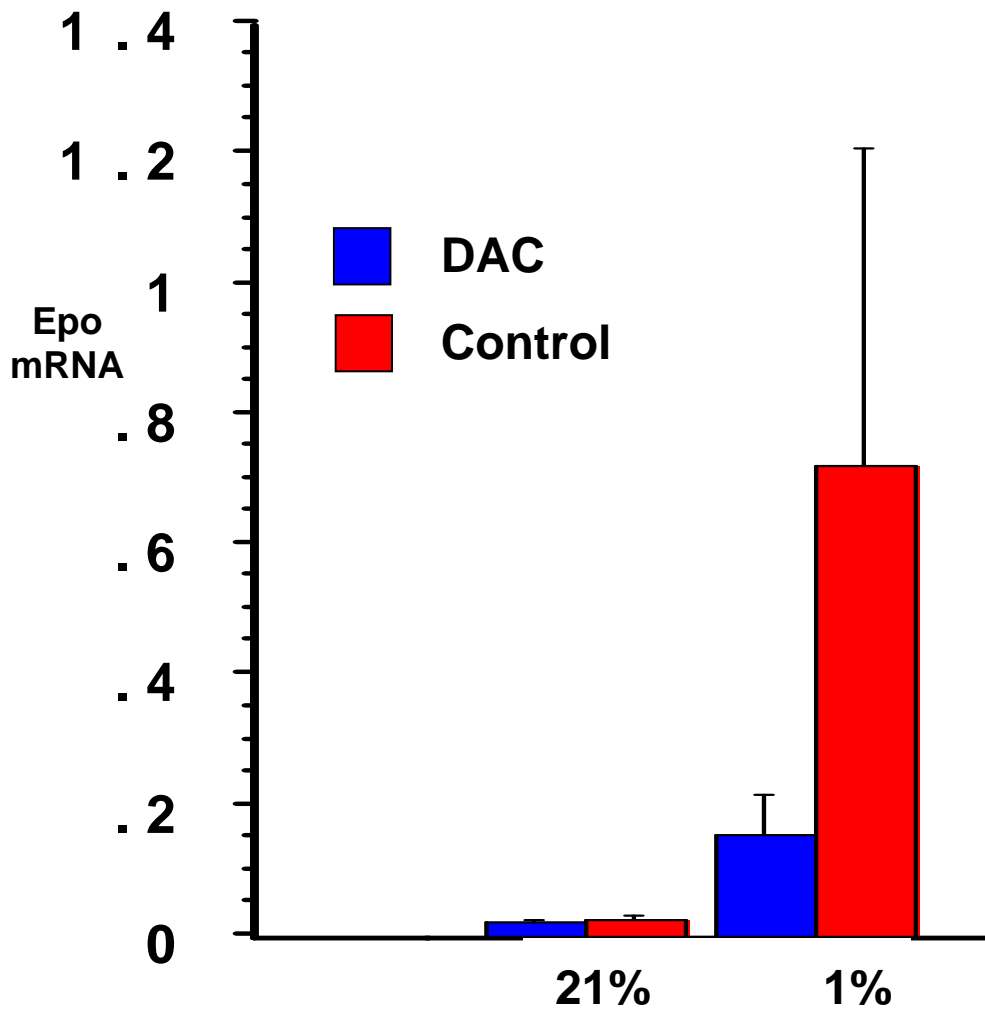
Figures

Figure 1. Liver



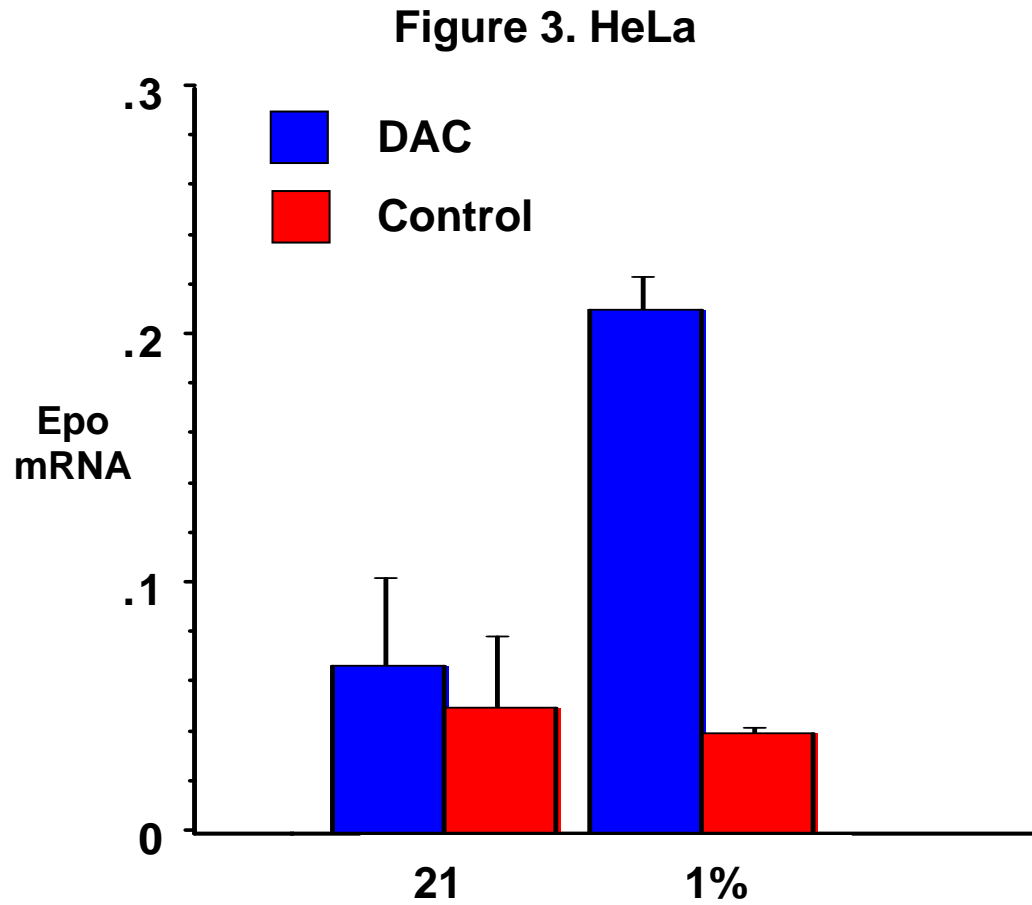
**Figure 1:** Epo mRNA concentrations were much greater in liver than kidney at all gestations tested ( $p < 0.001$ ). A twenty-fold increase in Epo mRNA concentrations occurred when liver samples were exposed to hypoxia ( $p = 0.001$ ), however this increase was not enhanced by pretreating the samples with DAC ( $p = 0.096$ ).

**Figure 2. Kidney**



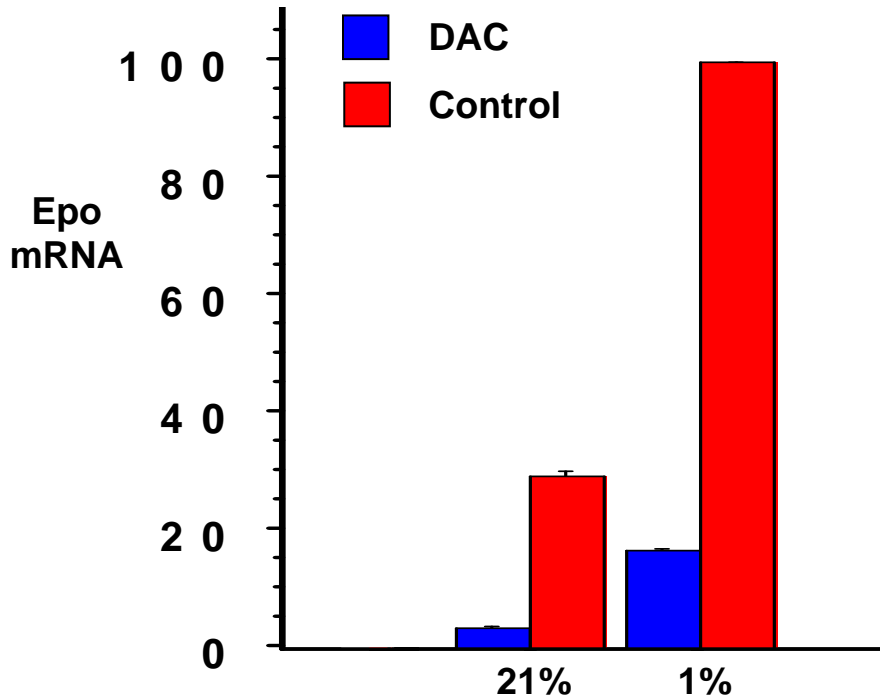
**Figure 2:** There was no statistical difference in Epo mRNA concentrations when kidney samples were exposed to hypoxia ( $p=0.168$ ). There was a four-fold increase in Epo mRNA concentrations when kidney samples were pretreated with DAC, however, this difference was not statistically significant.





**Figure 3:** HeLa cells were similar to kidney samples, in that they responded to pretreatment with DAC by increasing Epo mRNA concentrations under hypoxic conditions ( $p=0.006$ ).

**Figure 4. Hep 3B**



**Figure 4:** Epo mRNA concentrations in Hep 3B cells pretreated with DAC were lower under both normoxic and hypoxic conditions ( $p=0.004$  and  $p<0.001$ , respectively). The response to hypoxia appeared preserved in that pretreatment with either DAC or vehicle control resulted in increased Epo mRNA concentrations in response to hypoxia ( $p=0.0005$ , DAC pretreatment;  $p=0.0002$ , vehicle control).