EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 44, No. 11, 674-683, November 2012

Long-term and stable correction of uremic anemia by intramuscular injection of plasmids containing hypoxia-regulated system of erythropoietin expression

Jifeng Sun[1,5]*, Yarong Wang[2]*, Jie Yang[1,5]*, Dewei Du[1], Zhanting Li[1], Junxia Wei[3] and Angang Yang[4]

[1]Department of Nephrology
[2]Department of Radiology
[3]Clinical Research Center
Tangdu Hospital
Fourth Military Medical University
Xi'an 710038, China
[4]Department of Immunology
Fourth Military Medical University
Xi'an 710031, China
[5]Corresponding authors: Tel, 86-29-84777424;
Fax, 86-29-84777424; E-mail, jifeng-sun@medmail.com.cn (J.S.)
Tel, 86-29-84777424; Fax, 86-29-84777424;
E-mail, yangjie72029@yahoo.com.cn (J.Y.)
*These authors contributed equally to this work.
http://dx.doi.org/10.3858/emm.2012.44.11.076

Accepted 28 August 2012
Available Online 18 September 2012

Abbreviations: BUN, blood urea nitrogen; CMV IE, cytomegalovirus immediate-early basal gene promoter; CRF, chronic renal failure; Epo, erythropoietin; Hct, hematocrit; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia response element; pCMV-Epo, plasmids constructed by fusing human Epo gene to *CMV IE*; PGK, phosphoglycerate kinase; pHRE-Epo, plasmids constructed by fusing human *Epo* gene to the chimeric *PGK HRE* in combination with *CMV IE*; SCr, serum creatinine

Abstract

Relative deficiency in production of glycoprotein hormone erythropoietin (Epo) is a major cause of renal anemia. This study planned to investigate whether the hypoxia-regulated system of Epo expression, constructed by fusing Epo gene to the chimeric phosphoglycerate kinase (PGK) hypoxia response elements (HRE) in combination with cytomegalovirus immediate-early (CMV IE) basal gene promoter and delivered by plasmid intramuscular injection, might provide a long-term physiologically regulated Epo secretion expression to correct the anemia in adenine-induced uremic rats. Plasmid vectors (pHRE-Epo) were synthesized by fusing human Epo cDNA to the HRE/CMV promoter. Hypoxia-inducible activity of this promoter was evaluated first *in vitro* and then *in vivo* in healthy and uremic rats ($n = 30$ per group). The vectors (pCMV-Epo) in which Epo expression was directed by a constitutive CMV gene promoter served as control. ANOVA and Student's t-test were used to analyze between-group differences. A high-level expression of Epo was induced by hypoxia *in vitro* and *in vivo*. Though both pHRE-Epo and pCMV-Epo corrected anemia, the hematocrit of the pCMV-Epo-treated rats exceeded the normal ($P < 0.05$), but that of the pHRE-Epo-treated rats didn't. Hypoxia-regulated system of Epo gene expression constructed by fusing *Epo* to the HRE/CMV promoter and delivered by plasmid intramuscular injection may provide a long-term and stable Epo expression and secretion *in vivo* to correct the anemia in adenine-induced uremic rats.

Keywords: anemia; erythropoietin; gene therapy; hypoxia response element; uremia

Introduction

Anemia is a frequent complication of chronic renal failure (CRF). Insufficient production of glycoprotein hormone erythropoietin (Epo) is a main causative factor of uremic anemia. Currently, regular administration of recombinant erythropoietin (rEpo) and its newer derivatives, such as derivatives of rEpo-α and -β, is the main clinical treatment (Singh, 2008), and millions of patients have benefited from it. Yet recent studies revealed some adverse effects of this treatment, for example, mortality and cardiovascular complications due to the use of epoetin (Collins *et al*., 2000; Regidor *et al*., 2006) and pure red cell aplasia induced by newer derivatives (Casadevall *et al*., 2002; Bennett *et al*., 2004; Pollock *et al*., 2008). These thorny problems have not been settled so far.

Epo gene therapy is another treatment and still

under investigation at present. Previous studies proved that direct transfer of Epo gene into the host *via* either viral or nonviral means could allow sustained Epo secretion to correct the renal anemia (Maione *et al*., 2000; Rizzuto *et al*., 2000; Maruyama *et al*., 2001). But this transfer may lead to constant and high-level production of Epo and even to potentially lethal polycythemia, because the transferred *Epo* gene was not under the control of physiologic hypoxia-inducible factor-1 (HIF-1) (Johnston *et al*., 2003; Fabre *et al*., 2008). Temporal control systems of transgene expression were proved to be able to avoid deleterious Epo secretion (Richard *et al*., 2005). Yet these systems are complicated in repeated administration and calculating the doses of therapeutic gene.

Hypoxia is a natural physiological condition to regulate Epo expression. When reduced oxygenation of blood reaches the kidney, the Epo gene expression and protein secretion are increased by the fibroblasts of the renal cortex and outer medulla to increase erythropoiesis. The induction of Epo gene transcription in hypoxia needs HIF-1 binding to a hypoxia response element (HRE) lying 3' to the Epo gene (Frede *et al*., 2011).

HIF-1 is an oxygen-sensitive transcriptional activator. Its primary function is to mediate the adaptation to hypoxia in cells and tissues, leading to the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival (Ke and Costa, 2006). HIF-1 consists of a constitutively expressed subunit HIF-1β and an oxygen-regulated subunit HIF-1α. The stability and activity of the α subunit of HIF are regulated by its post- translational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation. In normoxia, hydroxylation of two proline residues and acetylation of a lysine residue at the oxygen-dependent degradation domain of HIF-1α trigger its association with pVHL E3 ligase complex, leading to HIF-1α degradation *via* ubiquitin- proteasome pathway. In hypoxia, the HIF-1α subunit becomes stable and interacts with coactivators such as cAMP response element-binding protein binding protein/p300 and regulates the expression of target genes (Ke and Costa, 2006).

HRE is a key regulatory DNA sequence that controls gene expression specifically in response to low oxygen concentrations (Semenza *et al*., 1996). HRE can be identified in the 5'- or 3'- flanking regions of various genes, including tyrosine hydroxylase, Epo, vascular endothelial growth factor (VEGF), and several glycolytic enzymes including phosphoglycerate kinase (PGK) (Goldberg and Schneider, 1994; Semenza *et al*., 1994; Ataka *et al*., 2003). Utilizing the property that HRE is responsible for binding to the α subunit of HIF-1 to stimulate transcription in hypoxia, Binley *et al*. developed a hypoxia control system similar to the natural one that can switch Epo gene expression on and off. And this system avoided deleterious Epo secretion and maintained long-term normalization of hematocrit (Hct) in anemic Epo-deficient Epo-TAg transgenic mice (Binley *et al*., 2002). Yet it was unclear whether this system would work well under the influence of uremia. This study was to investigate this question.

This study adopted a strategy of PGK HRE in combination with cytomegalovirus immediate-early (CMV IE) basal gene promoter to construct the hypoxia-responsive promoter (HRE/CMV). Because PGK HRE has a relatively higher responsiveness to hypoxia (Boast *et al*., 1999) and CMV IE promoter is a good basal promoter that has been widely used as a useful component of eukaryotic expression vectors (Boshart *et al*., 1985). This study fused human Epo (hEpo) gene to the HRE/CMV promoter to develop the plasmid vectors and employed intramuscular injection as gene delivery method because plasmid injection can be repeated without apparent immune response to the *DNA* vector (Terada *et al*., 2002). To know whether this chimeric HRE/CMV promoter would be activated in hypoxic conditions and whether this promoter could direct hEpo gene expression to correct the renal anemia, this study first evaluated the hypoxia-inducible activity of this promoter *in vitro* and then *in vivo* in uremic rat model.

Results

HRE/CMV promoter directs reporter gene expression in response to hypoxia *in vitro*

To assess whether HRE sequences of PGK gene could achieve hypoxic regulation in the context of a CMV IE basal promoter, we substituted the endogenous CMV IE enhancer with HRE sequences of PGK gene in the natural orientation (Figure 1A) and cloned the resulting chimeric HRE/CMV promoter into EGFP vector.

Green fluorescent assay for hypoxia-inducible expression of EGFP showed that pHRE-EGFP-transfected HeLa cells that were incubated in hypoxic conditions had impressive cytoplasmic fluorescence. Whereas mutated HRE construct-transfected cells incubated in both hypoxic and normoxic conditions did not show significant EGFP fluorescence, and pCMV-EGFP transfected cells incubated in both hypoxic and normoxic conditions had similar cytoplasmic fluorescence (Figure 1B).

To determine to what extent the HRE/CMV

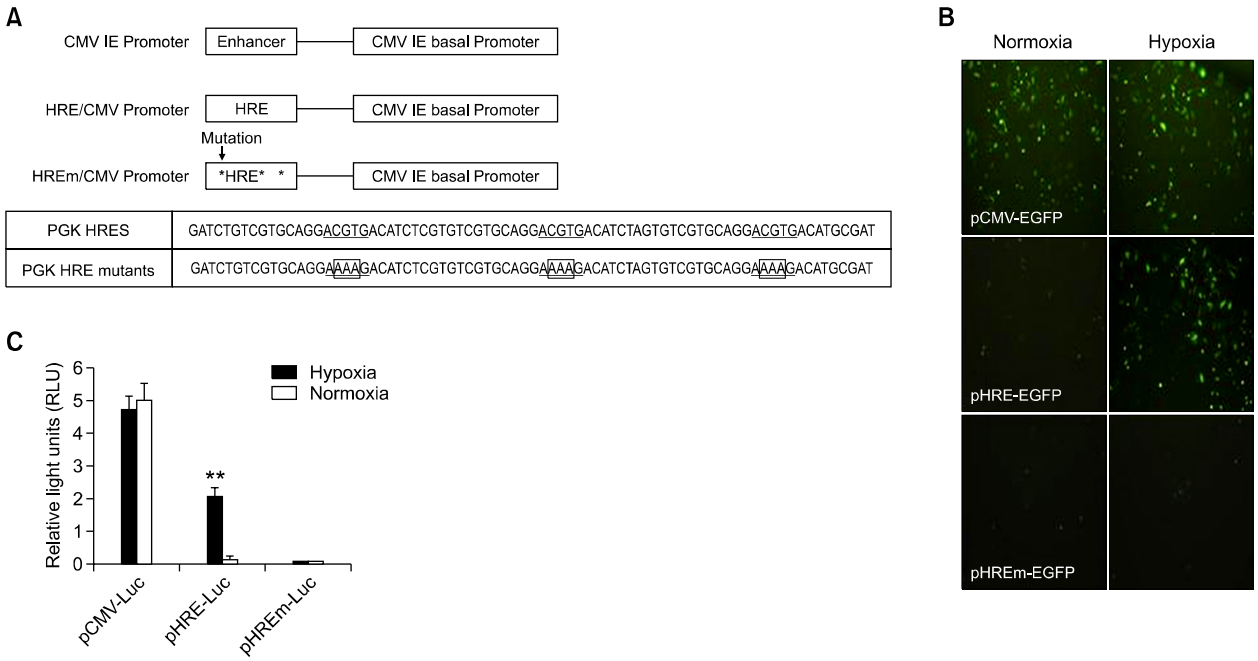

Figure 1. (A) HRE/CMV and HREm/CMV promoter were constructed by substituting the endogenous CMV IE enhancer with HRE sequences of PGK gene or mutated HREs in the natural orientation. In the chimeric HRE sequences of PGK gene and HRE mutant oligonucleotides, the core consensus sequence of HRE responsible for HIF-α binding is underlined. A 3-bp substitution in each of the HRE consensus sites in the trimer is boxed. 5'-end Bgl II and 3'-end Sgf I are shown in italics. (B) Cytoplasmic fluorescence in normoxic and hypoxic condition of HeLa cells transfected with pHRE-, pCMV- and pHREm-EGFP (fluorescence microscope Olympus BX-60; magnification $\times$ 200; 24 h after transfection). (C) Luciferase activity in normoxic and hypoxic condition of HeLa cells 24 h after transfected with pHRE-, pCMV- and pHREm-Luc. Data represent the means of two independent experiments, each carried out in triplicate. **stands for $P < 0.01$.

promoter would induce gene expression in response to hypoxia, we cloned the chimeric HRE/CMV promoter into firefly luciferase receptor vector to examine the luciferase activity. Renilla luciferase activity from pRL-CMV served as an internal control for transfection efficiency. As shown in Figure 1C, a hypoxic induction ratio of chimeric HRE/CMV construct was 20-fold greater in hypoxic conditions than in normoxic conditions, whereas no statistical difference was observed in mutant- or pCMV-Luc-transfected cells. The maximum level of the luciferase activity induced by hypoxia reached the 40% level that the constitutive CMV IE promoter achieved in normoxia. These data indicate that the HRE/CMV promoter can direct hypoxia-induced expression of reporter genes.

HRE/CMV promoter drives the secreting expression of Epo gene in response to hypoxia *in vitro*

Human Epo gene with an NH2-terminal signal peptide sequence was introduced to the downstream of the chimeric HRE/CMV promoter to create pHRE-Epo, likewise for pHREm-Epo and pCMV-Epo (Figure 2A). After the HeLa cells were transiently transfected with pHRE, pHREm-Epo, pHRE-Epo and pCMV-Epo, and then incubated in hypoxia or normoxia, the mRNA expression level of Epo in HeLa cells were detected using RT-PCR (Figure 2B) and the protein expression level of Epo using western blot (Figure 2C), showing that the Epo mRNA and protein levels in the pHRE-Epo transfected cells were obviously higher in hypoxia than in normoxia, that the Epo levels in the pHREm-Epo transfected cells were not detected both in hypoxia and in normoxia, and that the Epo levels in the pCMV-Epo transfected cells were the highest but had no difference between in hypoxia and in normoxia. These findings could be confirmed by immunofluorescence assay in which the Epo-expressing cells displayed bright fluorescence in the cytoplasm (Figure 2D).

The culture supernatant was harvested to examine the secreting expression of Epo in response to hypoxia. We found the *Epo* levels in the medium of HeLa-pHRE-Epo cells were much higher in hypoxia than in normoxia ($P < 0.01$), whereas the Epo secreted into the medium of HeLa-pCMV-Epo cells were at a similar level in both hypoxia and normoxia (Figure 2).

A growth factor-dependent cell line TF-1 cells were incubated together with the culture supernatants of HeLa-pHRE-Epo cells or HeLa-pCMV-Epo cells for

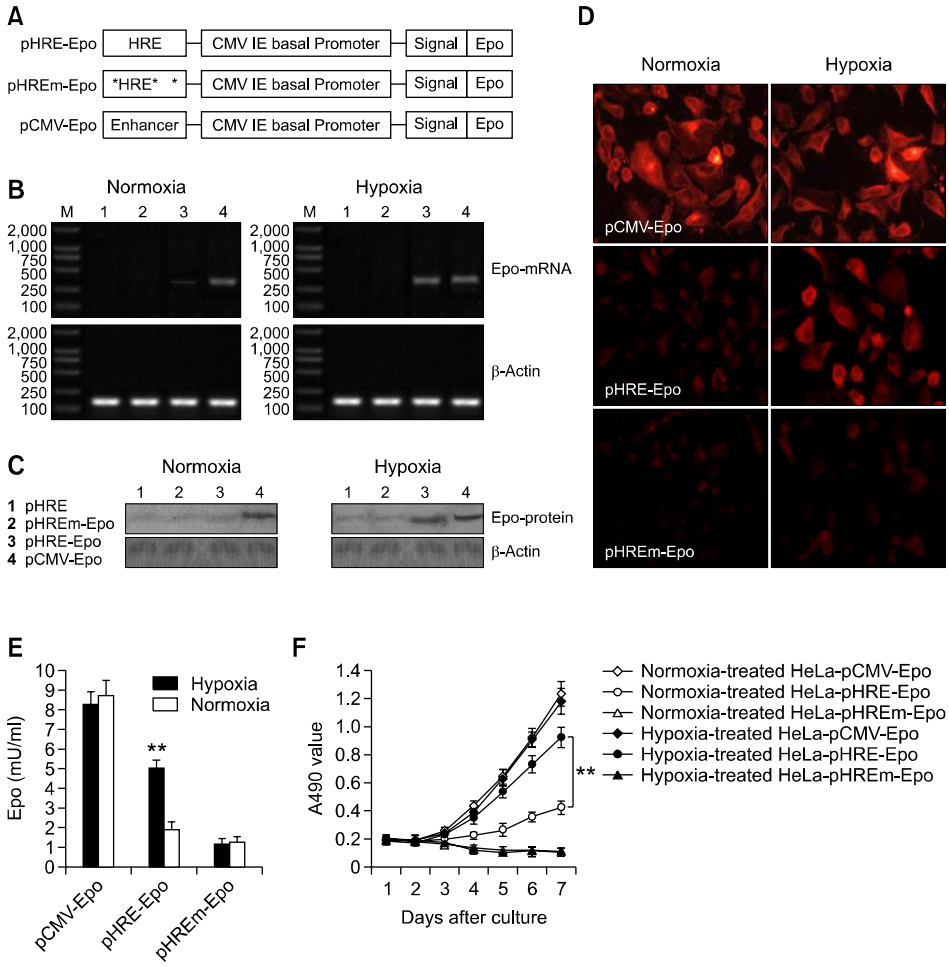

Figure 2. (A) Hypoxia-activated *Epo* construct was created by inserting the *Epo* gene with NH2-terminal signal peptide sequence into the downstream of the chimeric HRE/CMV promoter. (B) mRNA expression levels of Epo in HeLa cells transfected with pHRE, pHRE-Epo, pCMV-Epo and pHREm-Epo in normoxic and hypoxic condition. (C) Protein levels of Epo in HeLa cells transfected with pHRE, pHRE-Epo, pCMV-Epo and pHREm-Epo in normoxic and hypoxic condition. (D) Immunofluorescence of the cells transfected with pHRE-Epo, pCMV-Epo and pHREm-Epo in normoxic and hypoxic condition (the Epo-expressing cells showing bright fluorescence in the cytoplasm; magnification: × 400). (E) Secreting expression level of Epo in the culture supernatants of HeLa cells transfected with pHRE-Epo, pCMV-Epo and pHREm-Epo in normoxic and hypoxic condition was analyzed with Epo enzyme-linked immunosorbent assay. **stands for P < 0.01. (F) Viable TF-1 cells incubated with the supernatants of HeLa cell transfectants were assessed by MTT assay. At the 4th day, the A490 value of the hypoxia-treated Hela-pHRE-Epo is significantly higher than that of the normoxia-treated Hela-pHRE-Epo (P < 0.05), and at the 7th day this difference is the most obvious (*P* < 0.01). Data represent the mean values of two independent experiments, each carried out in triplicate. **stands for *P* < 0.01.

evaluating the biological activity of Epo expression *in vitro*. As shown in Figure 2F, TF-1 cells exhibited promoted survival and sustained proliferation in the medium of hypoxia-treated HeLa-pHRE-Epo cells, but in the medium of normoxia-treated HeLa-pHRE-Epo cells they didn't exhibit. TF-1 cells continually proliferated in the medium of both hypoxia- and normoxia-treated HeLa-pCMV-Epo cells. These findings indicate this promoter can drive the expression of biologically active Epo when induced by hypoxia *in vitro*.

In vivo study shows pHRE-Epo may correct uremic anemia safely and stably

The serum creatinine (SCr), blood urea nitrogen (BUN) and Hct of the rats were recorded once every two weeks during the experimental period (16 weeks), showing that compared with the healthy rats, the levels of SCr (Figure 3A) and BUN (Figure 3B) of all the uremic rats kept going up since the adenine diet started (*P* < 0.05). Morphologic examination demonstrated that the kidneys of uremic rats were enlarged and pale. Renal pathology showed us the crystal deposition in renal tubules and interstitium, renal tubular atrophy and

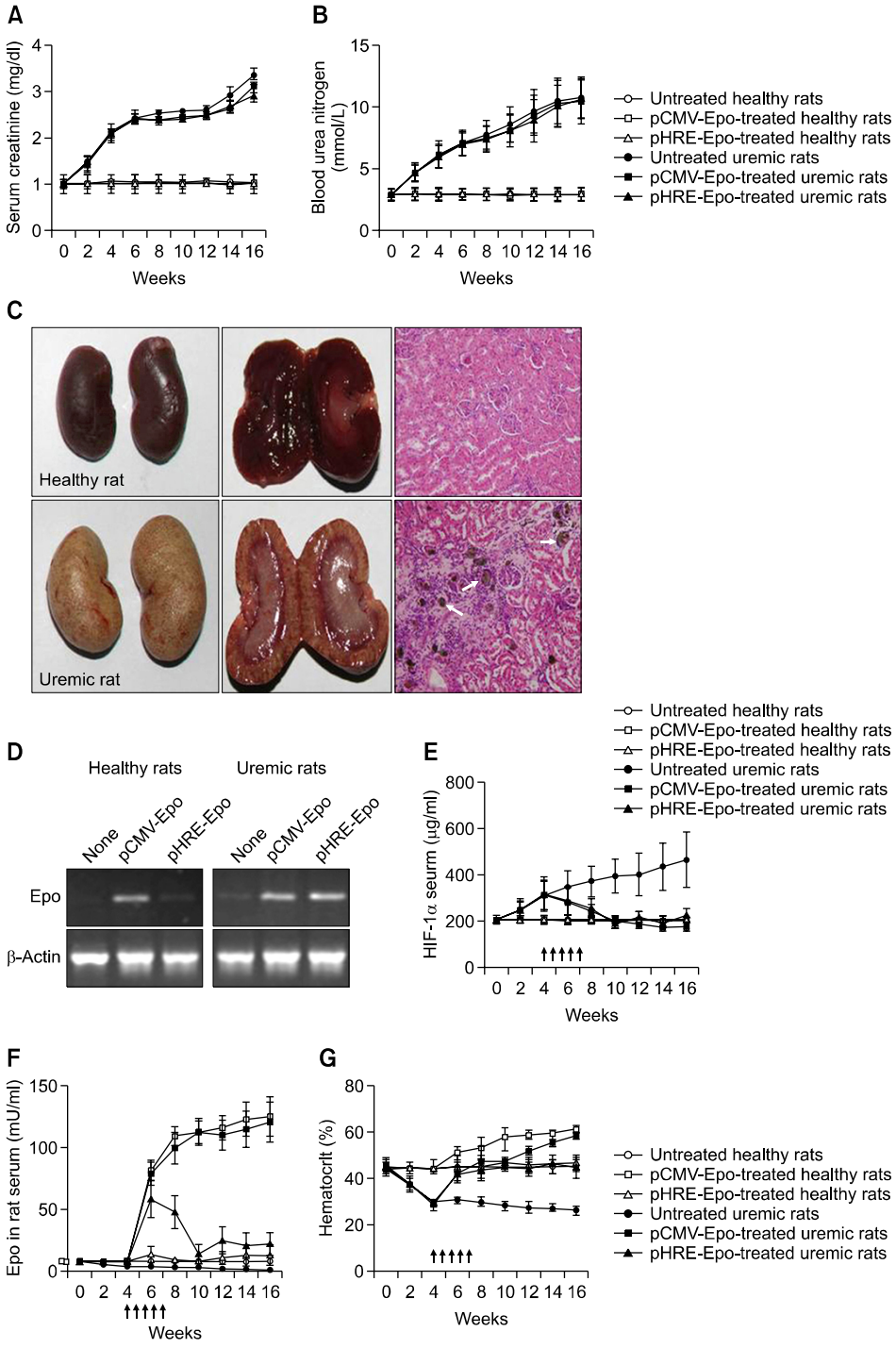

Figure 3. (A) Biweekly records of the SCr of all rats, uremic or healthy, show that the SCr of uremic rats goes up and keeps a high level since the 2nd week. (B) Biweekly records of the BUN of all rats show that the changes of BUN of uremic rats are similar to that of SCr of uremic rats. (C) The kidneys removed from the healthy and uremic rats display the obvious differences in the morphologic and histological observation in which the crystal depositions are marked with "white arrow". (D) One week after the end of intramuscular injection (in the 8th week), Epo expression in the muscular tissues around the injection location was detected. The muscular tissues (0.1 g) are collected from the untreated-, pHRE-Epo-treated-, pCMV-Epo-treated healthy and uremic *rats* (n = 2 per subgroup). (E) Biweekly records of *HIF-1*α in rat serum show no obvious alteration in healthy group. The HIF-1α levels of the uremic group keep going up in the first 4 weeks. But that of pHRE-Epo-treated or pCMV-Epo-treated uremic rats begins going down after the injection and gradually reaches the normal range. The HIF-1α levels of the untreated uremic rats elevate continuously. " ↑ " stands for the intramuscular injection of pHRE-Epo or pCMV-Epo. (F) The serum Epo of untreated healthy rat has no alteration but that of untreated uremic rats keep going down. In the pCMV-Epo-*treated* healthy or uremic rats, the serum Epo levels elevate continuously. In the pHRE-Epo-treated healthy rats, no change is observed. But in the pHRE-Epo-treated uremic rats, the serum Epo goes down before injection, goes up after injection and then fluctuates within a range. " ↑ " stands for the intramuscular injection of pHRE-Epo or pCMV-Epo. (G) Biweekly records of Hct demonstrate that only the Hct of pHRE-Epo-treated rats is better restored and maintained to normal physiological levels. " ↑ " stands for the intramuscular injection of pHRE-Epo or pCMV-Epo.

severe interstitial fibrosis in the renal cortex of uremic rats. Although the glomeruli of the uremic rats did not show crystalline deposition, but the glomeruli were sparse (Figure 3C). These indicate the construction of uremic model was successful.

The Epo mRNA expression in the hindlimb muscles was detected at the 8th week. The Epo mRNA expression was strong positive in both healthy and uremic pCMV-Epo-treated rats and at similar level between the healthy and the uremic. But in the pHRE-EPO-treated rats, the Epo mRNA expression was obviously higher in the uremic than in the healthy (Figure 3D). It means that the gene delivery by means of direct plasmid injection is efficacious

and that our constructed hypoxia-regulated system can respond to the hypoxia or normoxia condition *in vivo*.

The biweekly records of the HIF-1α level in rat serum (Figure 3E) demonstrated that the HIF-1α in untreated uremic rats kept going up after adenine diet, but that in pCMV-Epo-treated or pHRE-Epo-treated uremic rats was first obviously elevated since adenine diet and then descended to the normal level since plasmids injection. The biweekly records of Epo presented a trend roughly opposite to the HIF-1α in the untreated uremic rats and the pCMV-Epo-treated healthy and uremic rats, namely increased HIF-1α vs. simultaneously decreased Epo (Figure 3F). Correlation analysis proved a negative correlation between them ($P < 0.05$). But in the pHRE-Epo-treated uremic rats, the changes of Epo in rat serum had a similar tendency to that of HIF-1α, presenting a positive correlation ($P < 0.05$). This indicates that the hypoxia-regulated system can provide a physiologically switching on-off the Epo production *in vivo*.

The Hct level of pHRE-Epo-treated uremic rats went up continuously for 6 weeks after pHRE-Epo injection and then gradually reached a plateau within normal range and maintained the normal level for 6 weeks. The Hct level of the pCMV-Epo-treated rats, both uremic and healthy, were continuously and dramatically elevated and exceeded the normal range for 4 or 8 weeks after pCMV-Epo injection (Figure 3G). This indicates that the HRE/CMV-directed Epo plasmids can safely and stably correct the anemia *via* restoring and maintaining HCT to the normal physiological level for a long time.

Discussion

Creatinine is a break-down product of creatine phosphate in muscle and chiefly filtered out of the blood by the kidneys. Measuring SCr is a simple test used as an indicator of renal function (Delanghe *et al*., 1989). The BUN test is also a measurement of renal function. It is to measure the amount of nitrogen in the blood in the form of urea that is a by-product from metabolism of proteins by the liver and is removed from the blood by the kidneys. Hct is the volume percentage (%) of red blood cells in blood which is considered an integral part of a person's complete blood count results, along with hemoglobin concentration, white blood cell count, and platelet count (Purves, 2004). Our results of SCr, BUN and Hct in uremic group show the rat model of uremic anorexia induced by adenine-diet was successful. The experiments *in vitro* prove that the hypoxia-inducible system of Epo expression that we constructed can drive the Epo gene and protein expression in response to hypoxia, and those *in vivo* confirm that this system can correct the uremic anemia and, more importantly, can maintain the *Hct* within the normal range for long time in the uremic rats. These results are basically congruent with Binley's study though the vectors and animal models used by the two studies are different (Binley *et al*., 2002).

HRE is a key regulatory DNA sequence to control gene expression in response to hypoxia. Many previous studies reported that they treated multiple diseases by delivering therapeutic genes under the control of chimeric hypoxia-responsive promoters constructed by diverse HREs in combination with heterologous promoters, such as tumor killing by delivering the apoptotic genes or "suicide genes" encoding prodrug-activating enzymes (Wang *et al*., 2005; Greco *et al*., 2006), the treatment of cardiovascular disease by VEGF gene delivery (Lee *et al*., 2003), the protection of tissues against I/R injury by heme-oxygenase-1 gene (Tang *et al*., 2005; Pachori *et al*., 2006), and correction of anemia by Epo gene (Binley *et al*., 2002). Therefore it is reliable to utilize HRE to regulate target gene expression. We selected PGK HRE strategy to construct the hypoxia responsive promoter, because murine PGK HRE has a relatively better hypoxia response mechanism (Boast *et al*., 1999) and incorporating PGK HREs in gene delivery vectors can provide an on-off physiological switch that renders the transcription of therapeutic gene completely responsive to hypoxia/ischemia. Such a pattern of endogenous regulation of transgene expression is similar to natural mechanism.

Hypoxia control system utilizes a highly-conserved signaling machinery involving endogenous transcription factor HIF (Pachori *et al*., 2004). Although Epo gene expression is cell-type specific, most cell types can activate the HIF-1 pathway in response to hypoxia. And many genes active in a broad range of cell types and tissues contain HREs and can respond to stabilization of the HIF-1 in hypoxia. Besides kidney and liver, muscle tissues are also believed to be able to sense the hypoxia as oxygen delivery is reduced, and this may be sufficient to activate gene expression from a hypoxia-responsive promoter (Binley *et al*., 2002). Considering that the vascularity of skeletal muscle allowed for the distribution of secreted proteins and that skeletal muscle was easily targeted by injection in a clinical setting, we selected intramuscular injection as the method of gene delivery.

At present, the delivery of Epo gene is mainly dependent either on introducing the Epo gene into autologous cells *ex vivo* and transferring the modified

cells back into the individual (Bohl *et al*., 1997), or on directly transferring the Epo gene *in vivo* using an adenovirus vector, adeno-associated vector, or naked plasmid (Ye *et al*., 1999; Bohl *et al*., 2000). *Ex vivo* gene transfer is expensive and complicated to operate. Adenovirus vector induces the immunoresponse to adenovirus, which prevents long and stable gene expression in immunocompetent mice (Rivera *et al*., 1999). Plasmid injection, whereas, can be repeated without apparent immune response to the DNA vector (Terada *et al*., 2002), and has been successfully applied to deliver Epo gene into the muscles of mice to maintain long-term expression of Epo in the systemic circulation (Tripathy *et al*., 1996). Thereupon, naked plasmid injection becomes our preference although its transfection efficacy is lower than adenovirus vector's (Wolff *et al*., 1992). It was reported that the transfection rate of plasmid injection could be dramatically raised by means of *in vivo* electroporation (Maruyama *et al*., 2000; Terada *et al*., 2002). Yet we didn't use this technique, mainly because our primary purpose was to test whether our hypoxia-regulated system of Epo worked well *in vivo* or *in vitro*, rather than to contrive to raise the transfection rate of naked plasmid injection. Even if we didn't use this technique, our results of *in vivo* experiment confirmed that five consecutive injections of plasmids ensured the success of transfection.

Regulation of transgene expression is the utmost key factor for the safety and efficacy of gene transfer therapy. Scientists have developed the gene-switch system under pharmacological control of doxycycline (Bohl *et al*., 1998), rapamycin (Ye *et al*., 1999) and mifepristone (Terada *et al*., 2002). Comparing with them, the biggest advantage of the hypoxia control system is that it can switch transgene expression on and off without relying on the regular drug administration. Thus we think the hypoxia control system of Epo expression is more promising in the future clinical application, deserving more attention.

It has been proved that Epo may act as an angiogenic factor in malignant tumors (Ribatti *et al*., 2003b; Arcasoy *et al*., 2005; Jeong *et al*., 2008), and may stimulate proliferation and inhibit apoptosis of Epo-receptor-bearing tumor cells (Acs *et al*., 2001). Hypoxia may mediate the selection of neoplastic cells (Acs *et al*., 2003) and this may contribute to metastasis and treatment resistance of malignancies (Ribatti *et al*., 2003a). Whether the use of this hypoxia control system would induce, or on the contrary, inhibit tumor formation in uremic rats is still unclear. A recent report by Nairz *et al*. indicated that Epo-treatment had anti-inflammatory effect (Nairz *et al*., 2011). Whether this system would reduce the inflammatory response or protect against cardiac hypertrophy is still unclear. All these unsettled questions need more researches in future.

In sum, we constructed the chimeric HRE/CMV promoter by fusing the tandem repeats of PGK HRE to the CMV IE basal promoter, and constructed the hypoxia control system of Epo expression by fusing Epo gene to the HRE/CMV promoter. Our data confirm that the HRE/CMV promoter can sense the hypoxia in skeletal muscles and switch Epo expression on and off accordingly, and that intramuscular injection of plasmids containing this system provides a long-term physiologically regulated Epo secretion *in vivo* to correct uremic anemia efficiently and safely. Though it is promising to use this technique to treat uremic anemia, there are still some unsettled questions, deserving more attention in future.

Methods

Plasmids

We synthesized complementary oligonucleotides that contained HRE sequences of the flanking region of murine PGK gene and HRE mutants with 3-bp substitution in each of the HRE consensus sites in the trimer (Figure 1A). The annealed HREs oligos with HREs flanked by 5'-end Bgl II and 3'-end Sgf I overhangs were linked to the CMV IE promoter in the pCI-neo vector (Promega) to create pHRE/CMV. The chimeric pHRE/CMV promoter cassette was then cloned into pGL3-Basic (Promega) and pEGFP-C2 (Clontech) respectively. hEpo gene obtained by reverse-transcription PCR from fetal liver tissue was cloned into multiple clone sites in pHRE/CMV to create pHRE-Epo. The hEpo PCR product was cloned into the pUC18 plasmid (Panvera) and was subsequently removed as an XbaI-EcoRI fragment and cloned in to the pCI-Neo (Promega) NheI-EcoRI sites to create pCMV-Epo. All constructs were confirmed by DNA sequencing.

Cell culture, transfection and hypoxic treatment

Human cervical carcinoma cell line HeLa (obtained from the Lab of Professor Angang Yang, Fourth Military Medical University (Jia *et al*., 2003)) was maintained in RPMI1640 medium in which 10% fetal bovine serum had been supplemented. The cells were seeded in 24-well plates (density 3×10^4 cells/well) 24 h before transfection. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were transferred into hypoxic conditions (0.1% O_2, 5% CO_2, and 94.9% N_2) for transient expression. 24 h after transfection the cells were harvested.

Green fluorescent protein and luciferase assay

HeLa cells were transfected with recombinant EGFP plasmids and then they were incubated in hypoxic conditions and visualized by EGFP expression for green fluorescent assay. The cells co-transfected with Firefly and Renilla luci-

ferase vectors were incubated in hypoxia. The luciferase analyses were performed with Dual-Luciferase Reporter (DLR) Assay System (Promega) according to the manufacturer's protocol in a Turner Designs Luminometer TD-20/20 (Promega).

Immunofluorescence assay

Cells on coverslip were transfected with recombinant Epo plasmids and incubated in hypoxia. And then the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained using anti-human erythropoietin antibodies (R&D Systems) and cy3-conjugated IgG secondary antibodies (sigma).

RNA isolation and determination of Epo gene expression

Epo-mRNA was determined by RT-PCR. RNA was extracted from cells or tissues using the RNeasy RNA isolation kit (Invitrogen) and was reverse transcribed into cDNA using Superscript III reverse kit (Invitrogen). Primers for Epo were 5'-GGAAGAGGATGGAGGTCG-3' (forward primer) and 5'-GCAGTGATTGTTCGGAGTG-3' (reverse primer), and the size of the amplified fragment was 253 bp. Primers for β-actin of HeLa cells were 5'-GTCACCAACTG GGACGACA-3' (forward primer) and 5'-CACAGCCTGGAT AGCAACG-3' (reverse primer), resulting in a 192 bp product. Primers for β-actin of rats were 5'-CATTGTCACCAACTGG GACG-3' (forward primer) and 5'-GGTACATGCATCGGTA GGTC-3' (reverse primer), resulting in 190 bp product.

PCR was performed with PE2400 instrument (Perkin Elmer). Cycling conditions were: a) for RNA extraction form cell culture: 95°C for 5 min (1 cycle); 95°C for 30 s, 53°C for 30 s, 72°C for 45 s (25 cycles); 72°C for 7 min (1 cycle), and b) for RNA extraction form tissue: initial denaturation (95°C, 3 min), 40 cycles of denaturation (95°C, 10 s), annealing (60.5°C, 10 s), extension (72°C, 10 s), and a final extension (72°C, 10 min). PCR amplification products were analyzed in 1.5% agarose gels.

Western blot analysis and ELISA assay

Transfected cells were lysed in 50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. The samples (10 μg protein) were subjected to electrophoresis using a 15% SDS-polyacrylamide gel and the separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-human erythropoietin antibodies or β-actin antibodies (Sigma) and detected with an enhanced chemiluminescence system (Pierce). Epo levels in culture supernatants of the transfected cells were determined using the human Epo ELISA Kit (ExCell Biology).

Cell viability assay

TF-1 cells (from ATCC) were plated in 96-well dishes (density 5×10^3 cells/well) and then followed by incubation with culture supernatants that had been harvested from HeLa cells 24 h after transfection with recombinant Epo plasmids. 20 μl aliquots of MTT solution (5 mg/ml in PBS) were added to each well, followed by 4 h incubation and addition of 150 μl of DMSO (Sigma). Sunrise microplate reader (Tecan) tested A490 values. TF-1 cells without culture supernatants or incubated with culture supernatants of pCMV-Epo or pHRE-Epo transfectant were used as controls.

Experimental animals and *in vivo* study

The Ethics Committee of Fourth Military Medical University approved animal use (approval ID: 2009-LS-0617). Animal care and treatment were conducted in conformity with institutional guidelines that are in compliance with international laws and politics. 60 SD rats (180 ± 10 g, 6-8 weeks) were purchased from Laboratory Animal Center in Fourth Military Medical University and randomly classified into two groups, uremic and healthy group ($n = 30$ per group). Each group was further assigned into three subgroups, pHRE-Epo-treated, pCMV-Epo-treated and untreated subgroup ($n = 10$ per subgroup).

The whole experiment lasted 16 weeks during which all rats in healthy group were housed under standardized conditions in plastic cages (light-dark cycle 12/12 hrs, temperature 22 ± 2°C, humidity 50 ± 10%), had free access to tap water and fed with standard diet (provided by the Laboratory Animal Center in Fourth Military Medical University). The rats in uremic group had the same conditions of housing and water-drinking, but they were fed with 4 weeks of 0.75% adenine diet and then 12 weeks of standard diet. In this study, adenine diet was used to induce chronic renal failure and severe anemia based on the protocol and rationale of adenine diet of previous studies (Yokozawa *et al*., 1986; Ataka *et al*., 2003).

Biweekly blood sample harvests from rat tail vein (0.5-1 ml) were performed (totally 9 times during the experiment) for detection of SCr, BUN, Hct, and the levels of HIF-1α and Epo in rat serum. SCr and BUN were determined using Cobas Inegra 400 Plus automatic biochemical analyzer (Roche), and Hct using CA620 blood analyzer (Medonica). The levels of HIF-1α and Epo in rat serum were quantitatively measured using rat HIF-1α ELISA kit (Shanghai Kanu Biotechnology Co., Ltd., China) and ELISA Kit for Epo in rat serum (E90028Ra, Uscn Life Science Inc., US) respectively. Specific procedures were performed according to the manufacturers' directions.

From the 4th week on, 100 μl lipofectamine-encapsulated 50 μg pHRE-Epo and 50 μg pCMV-Epo were intramuscularly injected into the quadriceps of the left or right hindlimb of the pHRE-Epo-treated and the pCMV-Epo-treated subgroups respectively at 3-d intervals for 3 weeks (totally 5 injections). At the 8th week, namely one week after the end of the injections, the Epo mRNA expression in the muscles of hindlimb around the injection location were detected. At the 16th week, all rats were killed for morphologic examination of kidney and renal pathology. The pCMV-Epo-treated- and untreated-rats served as controls.

Statistical analysis

Data were presented as means ± SD. Statistical analysis was done using one way analysis of variance (ANOVA) for multiple samples, Student's *t*-test for comparing paired

sample sets, and correlation analysis for determining a relationship between two variables. $P < 0.05$ were considered statistically significant.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 30370622 and No. 30872400), the Project supported by the Key Science and Technology Program of Shaanxi Province, China (No. 2006K14-G1), the Natural Science Research Plan in Shaanxi Province of China (No. 2003C2052). We acknowledge Professor Ji-Feng Sun and An-Gang Yang who designed this experiment and Ya-Rong Wang who wrote this manuscript. Otherwise we are thankful to Jie Yang, De-Wei Du, Zhan-Ting Li, Jun-Xia Wei who performed the experiment with great effort. Ji-Feng Sun and Jie Yang had full access to all the data in the study and took responsibility for the integrity of the data, the accuracy of the data analysis.

References

Acs G, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K, Verma A. Erythropoietin and erythropoietin receptor expression in human cancer. Cancer Res 2001;61:3561-5

Acs G, Zhang PJ, McGrath CM, Acs P, McBroom J, Mohyeldin A, Liu S, Lu H, Verma A. Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. Am J Pathol 2003;162:1789-806

Arcasoy MO, Amin K, Vollmer RT, Jiang X, Demark-Wahnefried W, Haroon ZA. Erythropoietin and erythropoietin receptor expression in human prostate cancer. Mod Pathol 2005;18:421-30

Ataka K, Maruyama H, Neichi T, Miyazaki J, Gejyo F. Effects of erythropoietin-gene electrotransfer in rats with adenine-induced renal failure. Am J Nephrol 2003;23:315-23

Bennett CL, Luminari S, Nissenson AR, Tallman MS, Klinge SA, McWilliams N, McKoy JM, Kim B, Lyons EA, Trifilio SM, Raisch DW, Evens AM, Kuzel TM, Schumock GT, Belknap SM, Locatelli F, Rossert J, Casadevall N. Pure red-cell aplasia and epoetin therapy. N Engl J Med 2004;351:1403-8

Binley K, Askham Z, Iqball S, Spearman H, Martin L, de Alwis M, Thrasher AJ, Ali RR, Maxwell PH, Kingsman S, Naylor S. Long-term reversal of chronic anemia using a hypoxia-regulated erythropoietin gene therapy. Blood 2002;100: 2406-13

Boast K, Binley K, Iqball S, Price T, Spearman H, Kingsman S, Kingsman A, Naylor S. Characterization of physiologically regulated vectors for the treatment of ischemic disease. Hum Gene Ther 1999;10:2197-208

Bohl D, Naffakh N, Heard JM. Long-term control of erythropoietin secretion by doxycycline in mice transplanted with engineered primary myoblasts. Nat Med 1997;3:299-305

Bohl D, Salvetti A, Moullier P, Heard JM. Control of erythropoietin delivery by doxycycline in mice after intramuscular injection of adeno-associated vector. Blood 1998;92:1512-7

Bohl D, Bosch A, Cardona A, Salvetti A, Heard JM. Improvement of erythropoiesis in beta-thalassemic mice by continuous erythropoietin delivery from muscle. Blood 2000; 95:2793-8

Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B, Schaffner W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 1985;41:521-30

Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, Michaud P, Papo T, Ugo V, Teyssandier I, Varet B, Mayeux P. Pure red-cell aplasia and anti-erythropoietin antibodies in patients treated with recombinant erythropoietin. N Engl J Med 2002;346:469-75

Collins AJ, Ma JZ, Ebben J. Impact of hematocrit on morbidity and mortality. Semin Nephrol 2000;20:345-9

Delanghe J, De Slypere JP, De Buyzere M, Robbrecht J, Wieme R, Vermeulen A. Normal reference values for creatine, creatinine, and carnitine are lower in vegetarians. Clin Chem 1989;35:1802-3

Fabre EE, Bigey P, Beuzard Y, Scherman D, Payen E. Careful adjustment of *Epo* non-viral gene therapy for beta-thalassemic anaemia treatment. Genet Vaccines Ther 2008; 6:10

Frede S, Freitag P, Geuting L, Konietzny R, Fandrey J. Oxygen-regulated expression of the erythropoietin gene in the human renal cell line REPC. Blood 2011;117:4905-14

Goldberg MA, Schneider TJ. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J Biol Chem 1994;269:4355-9

Greco O, Joiner MC, Doleh A, Powell AD, Hillman GG, Scott SD. Hypoxia- and radiation-activated Cre/loxP 'molecular switch' vectors for gene therapy of cancer. Gene Ther 2006; 13:206-15

Jeong JY, Feldman L, Solar P, Szenajch J, Sytkowski AJ. Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells. Int J Cancer 2008;122:274-80

Jia LT, Zhang LH, Yu CJ, Zhao J, Xu YM, Gui JH, Jin M, Ji ZL, Wen WH, Wang CJ, Chen SY, Yang AG. Specific tumoricidal activity of a secreted proapoptotic protein consisting of HER2 antibody and constitutively active caspase-3. Cancer Res 2003;63:3257-62

Johnston J, Tazelaar J, Rivera VM, Clackson T, Gao GP, Wilson JM. Regulated expression of erythropoietin from an AAV vector safely improves the anemia of beta-thalassemia in a mouse model. Mol Ther 2003;7:493-7

Ke Q, Costa M. Hypoxia-inducible factor-1 (*HIF-1*). Mol Pharmacol 2006;70:1469-80

Lee M, Rentz J, Bikram M, Han S, Bull DA, Kim SW. Hypoxia-inducible *VEGF* gene delivery to ischemic myocardium using water-soluble lipopolymer. Gene Ther 2003;10: 1535-42

Maione D, Wiznerowicz M, Delmastro P, Cortese R, Ciliberto

G, La Monica N, Savino R. Prolonged expression and effective readministration of erythropoietin delivered with a fully deleted adenoviral vector. Hum Gene Ther 2000;11: 859-68

Maruyama H, Sugawa M, Moriguchi Y, Imazeki I, Ishikawa Y, Ataka K, Hasegawa S, Ito Y, Higuchi N, Kazama JJ, Gejyo F, Miyazaki JI. Continuous erythropoietin delivery by muscle-targeted gene transfer using *in vivo* electroporation. Hum Gene Ther 2000;11:429-37

Maruyama H, Ataka K, Gejyo F, Higuchi N, Ito Y, Hirahara H, Imazeki I, Hirata M, Ichikawa F, Neichi T, Kikuchi H, Sugawa M, Miyazaki J. Long-term production of erythropoietin after electroporation-mediated transfer of plasmid DNA into the muscles of normal and uremic rats. Gene Ther 2001;8:461-8

Nairz M, Schroll A, Moschen AR, Sonnweber T, Theurl M, Theurl I, Taub N, Jamnig C, Neurauter D, Huber LA, Tilg H, Moser PL, Weiss G. Erythropoietin contrastingly affects bacterial infection and experimental colitis by inhibiting nuclear factor-kappaB-inducible immune pathways. Immunity 2011;34:61-74

Pachori AS, Melo LG, Hart ML, Noiseux N, Zhang L, Morello F, Solomon SD, Stahl GL, Pratt RE, Dzau VJ. Hypoxia-regulated therapeutic gene as a preemptive treatment strategy against ischemia/reperfusion tissue injury. Proc Natl Acad Sci USA 2004;101:12282-7

Pachori AS, Melo LG, Zhang L, Solomon SD, Dzau VJ. Chronic recurrent myocardial ischemic injury is significantly attenuated by pre-emptive adeno-associated virus heme oxygenase-1 gene delivery. J Am Coll Cardiol 2006;47: 635-43

Pollock C, Johnson DW, Horl WH, Rossert J, Casadevall N, Schellekens H, Delage R, De Francisco A, Macdougall I, Thorpe R, Toffelmire E. Pure red cell aplasia induced by erythropoiesis-stimulating agents. Clin J Am Soc Nephrol 2008;3:193-9

Purves WK. *Life, the science of biology*, 7th Ed, 2004, Sinauer Associates ; W.H. Freeman and Co., Sunderland, Mass. Gordonsville, VA

Regidor DL, Kopple JD, Kovesdy CP, Kilpatrick RD, McAllister CJ, Aronovitz J, Greenland S, Kalantar-Zadeh K. Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. J Am Soc Nephrol 2006;17:1181-91

Ribatti D, Marzullo A, Nico B, Crivellato E, Ria R, Vacca A. Erythropoietin as an angiogenic factor in gastric carcinoma. Histopathology 2003a;42:246-50

Ribatti D, Vacca A, Roccaro AM, Crivellato E, Presta M. Erythropoietin as an angiogenic factor. Eur J Clin Invest 2003b;33:891-6

Richard P, Pollard H, Lanctin C, Bello-Roufai M, Desigaux L, Escande D, Pitard B. Inducible production of erythropoietin using intramuscular injection of block copolymer/DNA formulation. J Gene Med 2005;7:80-6

Rivera VM, Ye X, Courage NL, Sachar J, Cerasoli F, Jr., Wilson JM, Gilman M. Long-term regulated expression of growth hormone in mice after intramuscular gene transfer. Proc Natl Acad Sci USA 1999;96:8657-62

Rizzuto G, Cappelletti M, Mennuni C, Wiznerowicz M, DeMartis A, Maione D, Ciliberto G, La Monica N, Fattori E. Gene electrotransfer results in a high-level transduction of rat skeletal muscle and corrects anemia of renal failure. Hum Gene Ther 2000;11:1891-900

Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 1994;269:23757-63

Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem 1996;271: 32529-37

Singh AK. Anemia of chronic kidney disease. Clin J Am Soc Nephrol 2008;3:3-6

Tang YL, Qian K, Zhang YC, Shen L, Phillips MI. A vigilant, hypoxia-regulated heme oxygenase-1 gene vector in the heart limits cardiac injury after ischemia-reperfusion *in vivo*. J Cardiovasc Pharmacol Ther 2005;10:251-63

Terada Y, Tanaka H, Okado T, Shimamura H, Inoshita S, Kuwahara M, Akiba T, Sasaki S. Ligand-regulatable erythropoietin production by plasmid injection and *in vivo* electroporation. Kidney Int 2002;62:1966-76

Tripathy SK, Svensson EC, Black HB, Goldwasser E, Margalith M, Hobart PM, Leiden JM. Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. Proc Natl Acad Sci USA 1996;93:10876-80

Wang D, Ruan H, Hu L, Lamborn KR, Kong EL, Rehemtulla A, Deen DF. Development of a hypoxia-inducible cytosine deaminase expression vector for gene-directed prodrug cancer therapy. Cancer Gene Ther 2005;12:276-83

Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Hum Mol Genet 1992;1:363-9

Ye X, Rivera VM, Zoltick P, Cerasoli F, Jr., Schnell MA, Gao G, Hughes JV, Gilman M, Wilson JM. Regulated delivery of therapeutic proteins after *in vivo* somatic cell gene transfer. Science 1999;283:88-91

Yokozawa T, Zheng PD, Oura H, Koizumi F. Animal model of adenine-induced chronic renal failure in rats. Nephron 1986; 44:230-4