Enhancing the Potency and Longevity of Highly Valuable Peptides Using Gene Fusion

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***Abstract* - One major issue regarding the clinical use of many peptides is their short half-life in the body, due to the rapid clearance from the circulation. To overcome this issue, the carboxyl-terminal peptide of human chorionic gonadotropin β subunit was ligated to the coding sequence of follitropin, thyrotropin, erythropoietin and growth hormone. This peptide contains 28 amino acids and four *O*-linked oligosaccharide recognition sites. It was postulated that the *O*-linked oligosaccharides add flexibility, hydrophilicity and stability to the protein. Ligation of this peptide to the coding sequence of these hormones has no effect on receptor binding and *in vitro* bioactivity. However it is dramatically increased half-life and bioactivity *in vivo*. Interestingly, the new analogs of follitropin and growth hormone were found not immunogenic in human. Follitropin already passed successfully clinical trials phase III and approved by The European Commission for human use. In addition, our results indicated that long acting growth hormone is not toxic in monkeys and it passed successfully clinical trials phase II in adults. Other strategy to stabilize the peptide hormones was to convert the heterodimeric structure to a single chain by fusing the subunits to a single gene with or without the carboxyl-terminal peptide of human chorionic gonadotropin β subunit. The single peptide chains of human thyroptropin were active and have longer half-life in the circulation.**

Keywords - recombinant proteins; long acting; follitropin; thyrotropin; human chorionic gonadotropin; growth hormone; erythropoietin.

I. INTRODUCTION

The family of glycoprotein hormones consists of Thyrotropin (TSH), Lutropin (LH), Follitropin (FSH) and Chorionic Gonadotropin (CG). These hormones are heterodimers, consisting of the noncovalent association of a common α subunit with a unique β subunit that confers biological specificity to the hormone [1] [2]. The individual subunits have no known biological activity. Thus, formation of heterodimer is essential for activity [1] [2] [3]. The α subunit within a species has an identical amino acid sequence in all four members of this hormone family. The β subunit shares considerable amino acid homology with one another, indicating that most likely they evolved from a common precursor [4] [5] [6]. The subunits achieve their tertiary structures by the formation of internal disulfide bonds; 5 in the α subunit and 2-6 in the β subunits. The location of cystein residues, which determines the 3-dimensional structure of the subunits by predicating their folding, in the β subunits is highly conserved among the various hormones as well as different species [4].

The glycoprotein hormones activate the target cells via adenylate cyclase-linked receptors through binding to the membrane receptors. FSH stimulates follicular development in the ovary and gametogenesis in the testes. LH, acts primarily in promoting luteinization of the ovary and in stimulating Leydig cell function of the testes. CG maintains the corpus luteum in the ovary during pregnancy. TSH is a major regulator of thyroid hormone synthesis and secretion from the thyroid gland. The thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) regulate the synthesis and secretion of TSH from the pituitary [1] [3].

The subunits contain one (TSHβ and LHβ) or two (α, FSHβ and hCGβ) asparagine N-linked oligosaccharide chains [1] [2]. One unique structural difference among the subunits is the sequence of the carboxy terminus. TSH and LH subunits contain short hydrophobic stretches at their termini, deduced from gene sequencing [5]. However, the mature subunit does not contain the sequence due to a final deletion process. hCGβ subunit is distinguish from the other human β subunits in that it contains a unique 29 amino acid carboxyl-terminal peptide (CTP) bearing four *O*-linked oligosaccharide chains (Fig.1). It has been suggested that the *O*-linked oligosaccharide chains play an important role in the secretion of intact hCG from the cell, enhaced bioactivity and prolonged its circulating half-life *in vivo* [6]. Deletion of the *O*-linked oligosaccharide chains from hCG, didn't affect assemly of the subunits or secretion of the dimer from the cell and *in vitro* bioactivity. On the other hand, it was shown that truncated hCG without the CTP is 3 times less potent than intact hCG *in vivo* [7]. On the other hand, the *O*-linked oligosaccharide chains play a minor role in receptor binding and signal transduction. These findings indicate that the CTP of hCGβ and the associated *O*-linked oligosaccharides are not important

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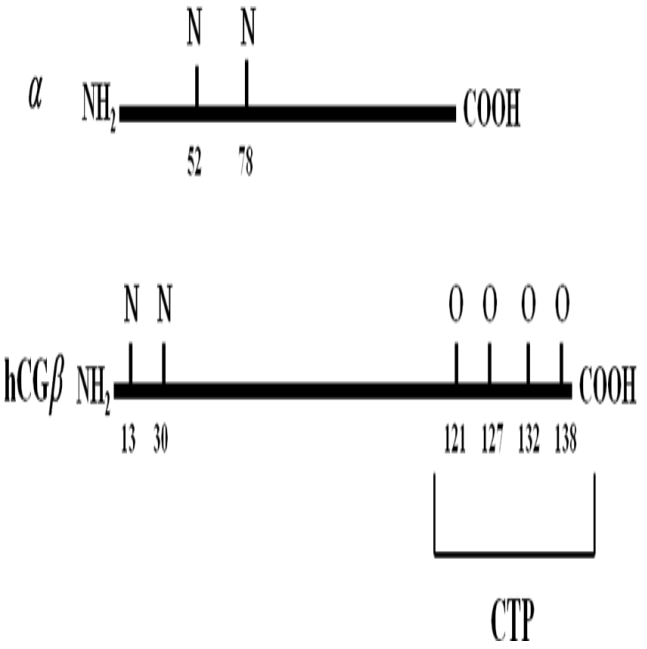

Figure 1. Human chorionic gonadotropin subunits. Localization of asparagine-linked carbohydrates (N) in both subunits are indicated. The CGβ subunit contain four sites of O-linked ologosaccharide chaines (O) in the CTP region.

for receptor binding or *in vitro* signal transduction, but are critical for *in vivo* bioactivity and half-life [6]

It was reported that the kidney is the main site of clearance for glycoprotein hormones [8]. On the other hand, much less hCG, which contains the CTP associatioted with the four *O*-linked oligosaccharide chains, is cleared by the kidney [9]. Other studies indicated that sialic acid plays an important role in the survival of glycoproteins in the circulation [1] [2]. It has been suggested that more negatively charged forms of glycoprotein hormones have longer half-lives, which may be related to a decrease glomerular filtration [10]. Thus, the presence of the CTP with its sialylated *O*-linked oligosaccharides may prolonged the circulating half-life of the hormone secondary to a decrease in renal clearance.

Erythropoietin (EPO) is a glycoprotein hormone produced primarily by cells of the peritubular capillary endothelium of the kidney [11] [13]. EPO is a member of an extensive cytokine family which also includes growth hormone, prolactin, interleukins 2 through 7, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), oncostatin-M, leukemia inhibitory factor and ciliaryneurotrophic factor. EPO production is stimulated by reduced oxygen content in the renal arterial circulation. Circulating EPO binds to EPO receptors on the surface of erythroid progenitors resulting in replication and maturation to functional erythrocytes by an incompletely understood mechanism.

The gene encoding human erythropoietin was cloned in 1985 leading to the production of recombinant human EPO (rhuEPO) [13]. The rhuEPO has been used successfully in a variety of clinical situations to increase production of red blood cells. Currently, this agent is licensed for use in the treatment of the anemia of renal failure, the anemia associated with HIV infection in zidovudine (AZT) treated patients, and anemia associated with cancer chemotherapy. Administration of rhuEPO has become routine in the treatment of anemia secondary to renal insufficiency where doses of 50-75 U/Kg given three times per week are used to gradually restore heamatocrit and eliminate transfusion dependency.

Human Growth Hormone (GH) is a member of family of closely related hormones that include prolactin and placental lactogen. GH regulates wide variety of physiological processes including growth and differentiation of muscle, bone and cartilage cells. GH is secreted by the somatotrophs of the anterior pituitary gland and acts on various tissues to promote growth and influence metabolism. GH antagonizes insulin effects by increasing hepatic gluconeogenesis and glycogenolysis by decreasing peripheral glucose consumption, effect that attributed to lipolytic effect. Disturbances of the carbohydrate metabolism can modulate the secretion of GH. The use of GH for the treatment of children with impaired linear growth has been accepted as an important therapeutic modality for many years. In addition, beneficial effects of GH replacement therapy in hypopituitary adults are well established. GH replacement therapy reduced body fat and increased body mass.

II. RESEARCH TOPICS

One major issue regarding the clinical use of glycoprotein hormones is their relatively short half-life *in vivo* due to their rapid clearance from the circulation when it's injected intravenously. Thus, the therapeutic protocol used in the treatment of glycoproteins, required frequent injections. The recommended therapy with rhuEPO is 2-3 times per week by subcutaneous or intravenous injections. Similarly, FSH or GH is injected daily. Therefore, we anticipated that ligation the CTP to the coding sequence of glycoprotein hormones will increase the half-life and bioactivity *in vivo*. This may reduce the number of injections per week. Therefore, it was hypothesized that FSH dimmer, EPO or GH containing the CTP would have a prolonged half-life and higher bioactivity *in vivo*. On the other hand, assembly of the hTSHβ and α-subunit is the rate limiting step in the production of functional heterodimer [3]. Thus, we hypothesized that converting hTSH to a single chain form could increase the half-life and expand the range of TSH structure – function studies. Here, we describe the construction of biologically active hTSH single chains with or without the CTP.

III. DESIGNINING GLYCOPROTEIN ANALOGS

To address the issue of the relatively short half-life of FSH, EPO and GH, the CTP of hCGβ was fused to the carboxyl-terminal of hFSHβ [14] [15], hEPO [16] and to the *N*- and *C*-terminal of human GH [17] coding sequences using overlapping PCR (Fig.2). Regarding the hTSH, the CTP was ligated to the C-terminal of β subunit. In addition the heterodimeric structure of hTSH was converted to a single chain by fusing the carboxyl end of hTSHβ subunit to the *N*-terminus of α-subunit in the absence or presence of the CTP sequence as a linker between the subunits (Fig. 3). It was noted that the *N*-and *C*- terminals of the hormones have no role in receptor binding or bioactivity. Therefore, it was hypothesized that ligation of these ends will not affect receptor binding or bioactivity. The designed chimeric genes were sequenced and ligateed into eukaryotic expression vectores. The plasmids were transfected into Chinese hamster ovary

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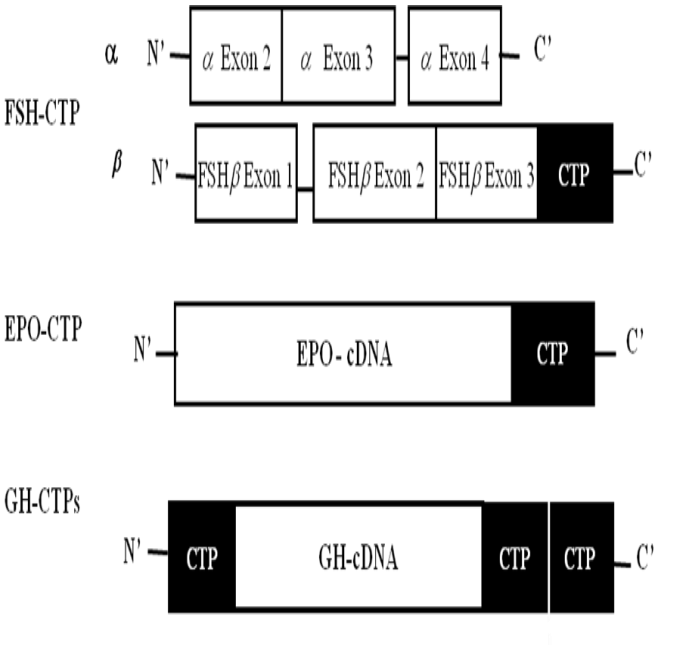

Figure 2. Construction of FSH-CTP, EPO-CTP and GH-CTP chimeric genes. The coding sequence of CTP was ligated to the 3rd end of hFSH*β gene or EPO* cDNA. One or two CTPs to be ligated to the N-terminal or to the C-terminal of GH cDNA, respectively.

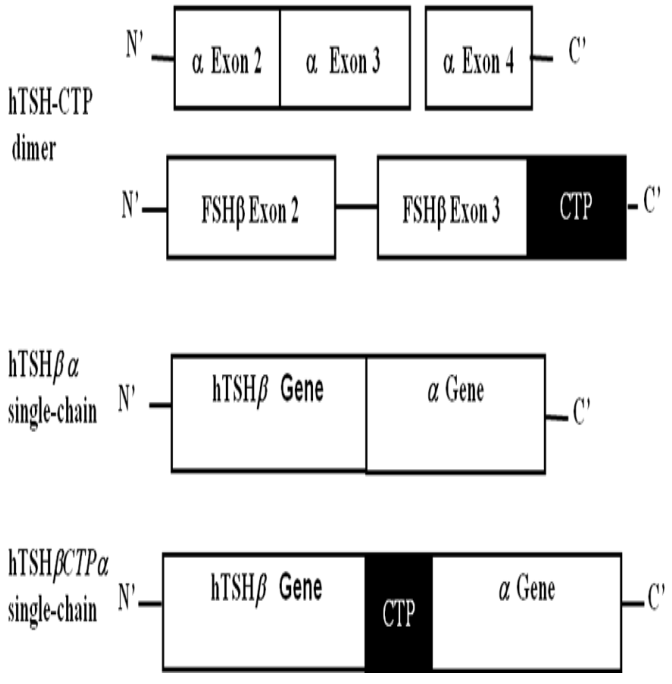

Figure 3. Construction of hTSH-CTP chimeric gene and single peptide chains with or without CTP as a linker between the subunits.

cells (CHO) and stable clones secreting the hormones were selected using selective antibiotics. Selected colonies resistant to antibiotics were harvested and screened for expression of the hormone. Collected media were concentrated and the hormones were detected immunoradiometric assay and a double antibody. Receptor binding was detected by radioreceptor assays. Bioactivity of the hormones was detected using *in vitro* and *in vivo* models.

IV. BIOACTIVITY OF THE DESIGNED ANALOGS

A. FSH

FSH induces aromatase enzyme in granulosa cells, therefore, signal transduction of the modified FSH dimer was assessed *in vitro* in the granulosa cell aromatase bioassay by measuring hormone-stimulated estrogen production. The detected steroidogenic activity of FSH-CTP chimeras was comparable to that of wild-type FSH. It was observed that ligation of CTP to the coding sequence of FSH*β* subunit, didn't affect receptor binding affinity or *in vitro* bioactivity. The *in vivo* bioactivity of wild-type FSH and the chimera was examined by determining ovarian weight augmentation and granulosa cell aromatase induction. It was found that ovarian weight increased significantly between animals treated with wild-type FSH and the FSH chimera. Treatment with FSH wild-type increased ovarian weight by 2 folds, where treatment with FSH-CTP increased ovarian weight by 3 folds. In addition, estrogen production by granulosa cells from chimera-treated rats increased 3- to 5-fold over that seen in rats treated with wild-type FSH. FSH-CTP was expressed in LDLD cells, a CHO cells mutated in the enzymes involved in addition of *O*-linked oligosaccharide chains. The results indicated that FSH-CTP lacking *O*-linked oligosaccharide have similar bioactivity of hFSH-WT (Fig.4).

Because the increased bioactivity of the chimeras may reflect a change in their *in vivo* longevity, the circulatory half-life of the hormones was detected. It seems that the clearance of the chimera is much slower than that of wild-type FSH; presumably. RIA determinations show that a high level of the chimera is still detectable in serum after 24 h and yet injected wild-type hFSH reaches basal level between 8 and 24 h.

The safety, pharmacokinetics and pharmacodynamics of FSH-CTP were studies in hypogonadotrophic hypogonadal male subjects as a phase I in multi-center study. The results indicated that FSH-CTP use is safe and does not lead to detectable formation of antibodies. Furthermore, pharmacokinetic and dynamic profile of FSH-CTP seemed to be promising. Compared with recombinant FSH – WT (Puregon), the half-life of FSH-CTP was increases 2-3 times [18] [19].

Further studies in *in vitro* fertilization (IVF) patients, indicated that a single dose of FSH-CTP is able to induce multifollicular growth comparing to daily injection of FSH-WT for 7 days [20]. According to the promising results described above in clinical trials, on January 28, 2010 the European Commission (EC) gave Merck & Co. marketing approval with unified labeling valid in all European Union Member States for FSH-CTP.

B. TSH

Because of TSH therapeutic potential, a longer acting analog of TSH was constructed by fusing the carboxyl-terminal extension peptide (CTP) of hCG*β* onto the coding sequence of TSH*β* subunit. When co-expressed either with alpha-subunit complementary DNA or alpha minigene in African green monkey (COS-7) or in human embryonic kidney (293) cells, the chimera was fully bioactive *in vitro* and

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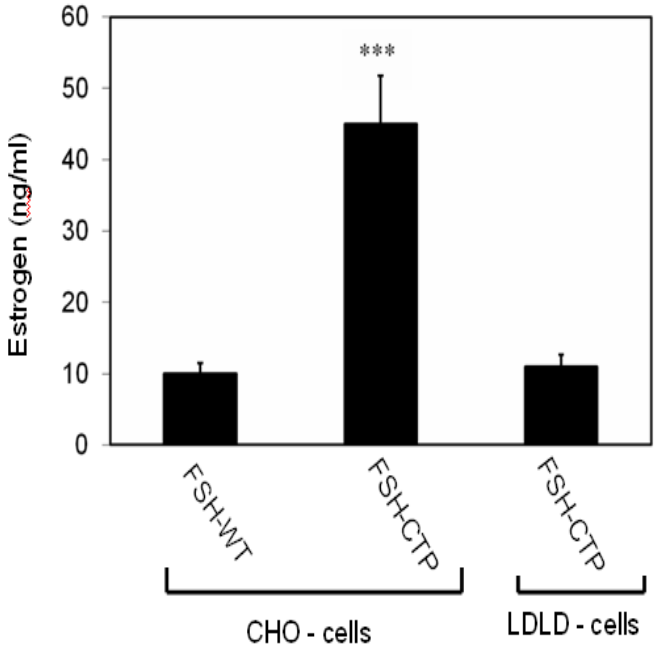

Figure 4. The effect of FSH on the level of estrogen in circulation of hypophysectomized rats. Recombinant FSH analogs were produced in CHO or LDLD cells'. ***P<0/001

exhibited enhanced *in vivo* potency associated with a prolonged plasma half-life. Ligation of the CTP to the C-terminal of TSHβ subunit did not affect the assembly and secretion of chimeric TSH [21]. Assembly of the glycoprotein hormone subunits is the rate-limiting step in the production of functional heterodimers [21]. To bypass this problem of dimerization of subunits, hTSHβ and α subunits were genetically fused in a single chain hormone with or without the CTP as a linker between the subunits. Single chains of hTSH retained a biologically active conformation similar to that of the wild-type heterodimer [22]. Moreover, it was found that the single peptide chains bind to the receptor with higher affinity and have higher bioactivity *in vivo* than the wild-type.

C. Erythropoietin

The CTP was ligated to the coding sequence at the C-terminal end of EPO. The *in vitro* biological activity of EPO-CTP was demonstrated by measuring their ability to stimulate proliferation of erythroid burst forming colonies (BFU-E) from human peripheral blood. BFU-E colonies were grown from blood of healthy donors using a microwell modification of the methylcellulose technique. The optimal formation of BFU-E colonies *in vitro* achieved by EPO-CTP was similar to that achieved by EPO-WT and rhuEPO using 1 U/ml of the protein. Receptor binding assay indicated that ligation of the CTP to the carboxyl-terminal of EPO has no significant effect on the affinity of the hormone to the receptor.

Further pharmacological evaluation of EPO-CTP, comparative pharmacodynamic studies of EPO-WT and commercial rhuEPO were performed in male C57BL mice (n=7/group) using different frequencies and wide dose range. The *in vivo* efficacy was obtained by measuring the mean values of haematocrit percentage in the blood. The results indicated that EPO-CTP is significantly (P<0.05) more efficient than EPO-WT when administered *IV* once a week with a dose of 200 mg/kg. EPO-CTP can successfully increase the haematocrit when administered once a week with a dose of 660 IU/kg. Once weekly dosing with the same concentration of commercial rhuEPO or EPO-WT was significantly (P<0.001) less efficient than once weekly dosing of EPO-CTP. An interesting observation is the ability of a single injection once a week of EPO-CTP (660 IU/kg) to increase the levels of haematocrit, whereas the same effect was achieved by administration of the same total dose of rhuEPO administered three times a week as 220 IU/kg per injection (Fig. 5).

These results indicated the importance of sustained blood levels, rather than total dose of EPO. These findings are consistent with the hypothesis that the ability of a single injection of EPO-CTP to increase haematocrit, results from its increased stability in the circulation. The increased biopotency of the chimera may reflect a change in their metabolic clearance *in vivo*. Detecting the half-lives of EPO analogs in mice indicated that a higher level of the chimera is still detectable in serum after 24 h. The half-life of EPO CTP is increased 2-3 folds comparing to EPO-WT. These data suggest that the mechanism of EPO metabolic clearance is affected by the presence of CTP [16].

D. Growth Hormone

Crystallographic studies indicated that *N*-terminal and *C*-terminal of GH are not important for binding of the hormone to its receptor. Therefore, CTP was fused to the *N*-terminal and *C*-terminal of hGH. The results indicate that ligation of CTP to the coding sequence of GH did not affect secretion of the chimeric protein into the medium. *In vivo* studies in hypophysectomized rats indicated that, bioactivity and pharmacokinetic parameters, MRT, AUC, Tmax, Cmax and half-life, of GH bearing the CTPs were dramatically enhanced. The estimated half-life of CTP-GH-CTP- CTP is increased by 4-5 folds comparing to Biotropin. These data suggest that the mechanism of GH metabolic clearance is affected by the presence of CTP [17]. Clinical trials phase I and phase II of GH-CTPs indicated that this peptide is safe for use and clinical trials of phase III is in the way.

V. CONCLUSIONS

Ligation of the CTP bearing four sites for *O*-linked oligosaccharide chains to different proteins indicated that the *O*-linked glycosylation recognition sites of the CTP are preserved. Moreover, this ligation is not involved in secretion, receptor binding and *in vitro* bioactivity. However, both the *in vivo* bioactivity and half-life in circulation of proteins bearing the CTP were significantly enhanced. Proteins containing the CTP could serve as long acting agonists for clinical use. This strategy may have wide applications for enhancing the *in vivo* bioactivity and half-life of diverse proteins. On the Other hand, converting the hetrerodimeric structure of the hormone to a single-chain polypeptide chain did not affect receptor binding affinity and bioactivity. However, this strategy is important for designing of new analogs of heterodimeric proteins.

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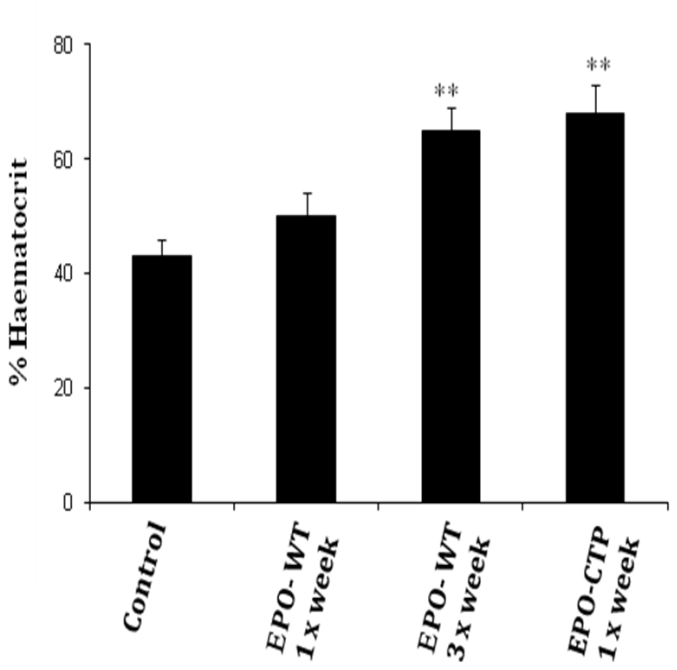

Figure 5. Ability of a single IV injection of EPO-CTP vs 3 IV injections of rHuEPO to increase haematocrit levels in mice. ICR mice (n=7/group) received a single IV injection/week for three weeks of EPO-CTP (660 IU/ kg) or wild-type rHuEPO (220 IU/kg) that were injected IV three times a week for three weeks. Control animals were injected IV with F-12 medium free of serum. Blood samples were collected three times a week and haematocrit levels were detected. Each point represents the group average of haematocrit (%) ± SE. **P<0/01.

VI. ACKNOWLEDGMENTS

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