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Peginesatide and Erythropoietin Stimulate Similar Erythropoietin Receptor-Mediated Signal Transduction and Gene Induction Events

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Abstract

Peginesatide is a synthetic, PEGylated, peptide-based erythropoiesis-stimulating agent that is designed and engineered to stimulate specifically the erythropoietin receptor dimer that governs erythropoiesis. Peginesatide has a unique structure that consists of a synthetic peptide dimer (with no sequence similarity to erythropoietin) conjugated to a 40 kDa PEG moiety. Peginesatide is being developed for the treatment of anemia associated with chronic kidney disease in dialysis patients. To compare signaling effects of peginesatide to recombinant human erythropoietin (rHuEPO), dose-dependent effects on protein phosphorylation and gene expression were evaluated using phosphoproteomics, quantitative signal transduction analyses and gene profiling. Following stimulation with peginesatide or rHuEPO, cell lysates were prepared from UT-7/EPO cells. LC-MS/MS and MesoScale arrays were used to quantify phosphorylation events. Transcriptional changes were analyzed using microarrays and quantitative RT-PCR. Peginesatide and rHuEPO were found to regulate the tyrosine phosphorylation of an essentially equivalent set of protein substrates, and modulate the expression of a similar set of target genes. Consistent with their roles in stimulating erythropoiesis, peginesatide and rHuEPO regulate similar cellular pathways.

Keywords

Erythropoiesis; signal transduction; gene expression; peginesatide; erythropoietin

Introduction

Recombinant human erythropoietin (rHuEPO) was approved two decades ago as a replacement therapy for the deficient production of erythropoietin in chronic kidney disease patients. Anemia associated with chronic kidney disease is debilitating, and before rHuEPO was available, many of these patients required frequent red cell transfusions to increase their hemoglobin concentrations. Due to its short 6-8 h plasma half-life [1], rHuEPO is administered three times per week to increase red blood cell levels. In the clinic, this becomes time and labor intensive due to frequent dose adjustments that can lead to hemoglobin variability. Hence, there have been various efforts to develop erythropoiesis stimulating agents (ESAs) that are longer-acting, or more conveniently administered [2].

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EPO derivatives such as darbepoetin and methoxy polyethylene glycol-epoetin beta rely on post-translational modifications to rHuEPO to enhance plasma half-life. Darbepoetin incorporates additional N-linked glycosylation sites, allowing for a dosing regime of every 1-2 weeks. Methoxy polyethylene glycol-epoetin beta is PEGylated to further increase plasma half-life and dosing intervals.

Another approach to develop novel ESAs involved the use of phage display technology to discover small EPO mimetic peptides (EMPs) that bind the EPOR with high affinity. One such peptide, EMP1, which lacks EPO sequence similarity, was shown to possess EPOR agonist activity [3, 4]. An X-ray crystal structure of EMP1 complexed with the EPOR extracellular domain demonstrated that EMP1 forms a tight, non-covalent dimer bound to two EPOR chains like EPO itself, but at a different overlapping binding site [5]. EMP1, however, exhibits only weak *in vivo* activity. Subsequent extensive evaluations of chemical analogues and architectural modifications (including dimerization and PEGylation) resulted in an optimized erythropoiesis stimulating agent (ESA) known as peginesatide (formerly known as Hematide TM). Peginesatide has been shown to stimulate sustained erythropoiesis *in vivo* allowing for once monthly dosing, and has recently been approved for the treatment of anemia associated with chronic kidney disease in patients on dialysis [6–9].

Based on the novel features of diverse ESAs currently under investigation, it is of considerable interest to better understand their mechanistic similarities and differences. This includes, in particular, how different structures may contribute to different *ex vivo* and *in vivo* properties. While much has been learned about some of the primary signaling pathways that rHuEPO stimulates in erythroid cells, much of this information was generated before more global approaches to signaling were readily available. We previously showed that peginesatide stimulated some of the same major signaling pathways as rHuEPO, including the activation of JAK2, STAT5A, STAT5B, ERK1/2, and AKT [6]. Here, we extend these analyses to include global phosphoproteomic and transcriptional studies in human erythroid progenitor UT-7/EPO cells in order to compare rHuEPO and peginesatide signaling on a wider canvas. Overall, we find predominant similarities despite peginesatide's unique structure (as a PEGylated, homodimeric peptide with no sequence homology to rHuEPO), but consistent with their shared roles in driving erythropoiesis.

Materials and methods

Phosphoscan analyses

The EPO-responsive, human erythroid cell line UT-7/EPO was obtained from Dr. Norio Komatsu (University of Yamanashi, Japan) and maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 1 U/mL rHuEPO (Procrit, epoetin alfa, Amgen, Thousand Oaks, CA), 2 mM GlutaMax-I, and 100 U/mL penicillin/100 µg/mL streptomycin. Unless otherwise stated, tissue culture reagents were obtained from Invitrogen Technologies (Carlsbad, CA). Cells (5×10^8) were washed and resuspended in IMDM, 10% FBS, 2 mM GlutaMax-I, 100 U/mL penicillin/100 µg/mL streptomycin without rHuEPO ("starvation medium", SM) and cultured for 16-18h (37 °C, 5% CO₂). Cells were then stimulated with 10 U/mL EPO, 10 nM peginesatide, or vehicle for 15 minutes at 37 °C. Cells were pelleted, lysed for 10 minutes on ice in urea lysis buffer (20 mM HEPES, pH 8.0, 9 M urea, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerol phosphate) and flash frozen at –80°C.

Upon thawing, lysates were sonicated, reduced, carboxyamidomethylated and digested with either trypsin or GluC. For GluC, an AQUA phosphotyrosine peptide from human CDC2 (CO4-167, Y15, IGEPTY*GVvYK) was synthesized, purified and used as an internal standard for intensity normalization. For both trypsin and GluC digests, peptides were then

separated from non-peptide material by solid-phase extraction with Sep-Pak C18 cartridges. Lyophilized peptides were re-dissolved, and phosphorylated peptides were isolated using an immobilized anti-phospho-tyrosine antibody (Cell Signaling Technology, Danvers, MA, catalog # 9411). Peptides were eluted from the antibody-resin (in a total volume of 100 μ L) in 0.15% trifluoroacetic acid, and concentrated with PerfectPure C18 tips prior to LC-MS analysis.

For mass spectrometry, peptides were loaded directly onto a 10 cm $\times$ 75 μ m PicoFrit capillary column packed with Magic C18 AQ reversed-phase resin. The column was developed with a 45 minute linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL/min. Tandem spectra were collected using an LTQ-Orbitrap hybrid mass spectrometer, top-ten method, dynamic exclusion repeat count of 1, and a repeat duration of 30 seconds. MS spectra were collected in an Orbitrap component, and MS/MS spectra were collected in the LTQ. MS/MS spectra were evaluated using TurboSEQUENT in the Proteomics Browser package (v. 27, rev. 13). Searches were performed against the National Center for Biotechnology Information (NCBI) human database, (September 05, 2006 release) containing 34,180 protein sequences in forward- and reversed-sequence directions. The false positive assignment rate was approximated via the ratio of reversed database assignments vs. forward database assignments after filtering initial SEQUEST search results based on XCorr (> 1.5), mass accuracy (± 10 ppm), and the presence of the expected sequence motif. The mass accuracy range was narrowed further based on the XCorr-mass error plot for each experiment.

Electrochemiluminescent assays of EPO- and ESA- modulated phosphoproteins

UT-7/EPO cells were washed and resuspended in SM medium, and cultured for 16-18 hours. Cells were then exposed to a dose response of peginesatide or rHuEPO for varied times, and lysed in an equal volume of 2X lysis buffer (Cell Signaling Technology, catalog #9803) for 1 hour at 4 $^{\circ}$ C. Cell lysates were analyzed for protein phosphorylation using MesoScale Discovery arrays according to manufacturer's protocols (Meso Scale Discovery, Gaithersburg, MD)

Gene expression analyses

UT-7/EPO cells were washed, resuspended in SM medium, and cultured for 16-18 hours. Cells were then treated with vehicle, peginesatide, or rHuEPO at varied concentrations. At defined intervals, cells were pelleted by centrifugation for 5 minutes prior to RNA isolation. RNA was isolated using the ToTALLYTM RNA kit (Ambion, Austin, TX). RNA quality and quantity assessed on an Agilent 2100 Bioanalyzer (Palo Alto, CA) using capillary electrophoresis prior to hybridization to a GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA). Analysis was performed using the Affymetrix MAS 5.0 algorithm. Statistical significance was determined using the one-way ANOVA model. To determine if gene induction differed between treatment groups, a two-sample t-test was carried out followed by multiplicity correction to control the false discovery rate (FDR) at 0.05. Multiplicity correction methods were conducted using Partek Genomic Solutions 6.2 Software (Partek Inc., St. Charles, MO), Matlab 7 (Mathworks Inc., MA), and GCOS 1.3 (Affymetrix Inc., CA). Principal Component Analysis was performed using the software package Partek Genomic Solutions 6.2 (Partek Inc., St. Charles, MO). Principal Component Analysis extracts relevant information from complex data sets by reducing dimensionality from thousands to only a handful of the most informative components. The heat map was created using Matrix2png (<http://www.bioinformatics.ubc.ca/matrix2png/>). Subsequent gene set pathway analyses were conducted using Rotation Gene Set Testing (ROAST).[10]. Significantly upregulated pathways were identified using a Benjamini-Hochberg FDR filter. Pathway descriptions can be found at <http://www.broadinstitute.org/gsea/msigdb/>

[collections.jsp](#). Microarray and bioinformatics analysis was conducted at Asuragen, Austin, TX.

Quantitative RT-PCR

RNA (as prepared with the RNeasy® 4-PCR RNA isolation kit) (Ambion) was reverse transcribed (200 ng) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Alternatively, cDNA was made using the TaqManFast Cells-to-CT kit (Applied Biosystems, Foster City, CA). PCR utilized thermal cycling conditions specified by the manufacturer. Data analysis was conducted using software provided with the StepOnePlus® system. Fold-changes in gene expression for treated samples compared to vehicle controls were calculated after normalization to the endogenous housekeeping gene HPRT1.

Results

Peginesatide and rHuEPO effects on global phosphorylation events

A phosphoproteomics approach was adopted to provide for broad comparisons of the signaling pathways modulated by rHuEPO and peginesatide. In particular, experiments focused on identifying tyrosine phosphorylation events stimulated in UT-7/EPO cells, a rHuEPO-dependent model that has been extensively used for characterizing EPO action. Following 18 hours of rHuEPO-withdrawal, UT-7/EPO cells were treated with rHuEPO or peginesatide for 15 minutes. Cell lysates then were prepared and processed for LC-MS/MS analysis by treatment with trypsin and immunoprecipitation of tyrosine phosphorylated peptides with an immobilized anti-phosphotyrosine antibody. Overall, 242 non-redundant phosphopeptides derived from 164 parent proteins were identified via these LC-MS/MS experiments. Label free quantification was performed from the peak intensities of the MS channel to provide the relative abundance of the identified phosphopeptides. Results of these analyses reveal dynamic activation of tyrosine phosphorylation events downstream of the activated EPOR, with 31 phosphopeptides showing an increase in abundance of more than 2.5 fold with both rHuEPO or peginesatide treatment as compared to vehicle controls (Table 1).

Many proteins that previously have been reported to be phosphorylated on tyrosine in response to EPOR ligation were presently observed, including JAK2, STAT5A, STAT5B, STAT3, GAB1, SHC1, CBL, CRKL, SHIP1, SHIP2, SHP2, PLC γ , VAV1 and ERK1/2 [11–21]. As expected, some of the largest changes in phosphorylation were observed to occur in the Ras/Raf/MAP kinase and the JAK/STAT pathways consistent with known erythropoietin-activated pathways (Table 1).

The above analyses were also repeated using the protease GluC (instead of trypsin) to create an orthogonal set of phosphopeptides for analysis. Here, 205 non-redundant phosphopeptides were identified with 48 increased more than 2.5 fold upon ESA treatment (Table 1). In this digest, the phosphorylation of the most C-terminal tyrosine site of the EPOR at position 504 was detected. We did not detect other EPOR phosphorylation sites most likely because method compatible peptides were not produced in digests (and/or because of the low level of EPOR expression). Several additional known downstream mediators of the EPOR signal transduction also were detected, including PI3 kinase, STAT1, STAT5B, p38, and GRB2 (Table 1) [18, 22–25].

Within data from combined experiments, several previously unpublished phosphorylation sites in known downstream signaling mediators were discovered. Additionally, many new proteins were found to be tyrosine phosphorylated in response to ESA treatment. The former category includes the phosphorylation of Y682 of STAT5B, Y183 of GAB1 and GAB3,

Y833 of PLC γ 1, and the recently identified residue Y52 of GRB2 (Table 1). Notable new proteins include the protein kinases FRK and Sgk269, the tyrosine phosphatase PTPN18, the transcription factor KLF5, and the adapter CD2AP (Table 1). The proteins Afadin, protocadherin, ezrin, and spectrin that are associated with cytoskeletal structure and cell-cell interactions also were modulated, as was the mitochondrial enzyme ornithine carbamoyltransferase (Table 1). There were also three proteins of unknown function whose phosphorylation was induced by both rHuEPO and peginesatide. Further confirmation of these phosphorylation events, and evaluation of functions within erythroid progenitors are currently under investigation.

Peginesatide and rHuEPO actions compared via quantitative signal transduction studies

To further compare rHuEPO and peginesatide modulated signal transduction events, we employed the Meso Scale Discovery (MSD) platform to specifically evaluate the phosphorylation of JAK2, STAT3, STAT5, ERK1/2 (MAPK1/3), GSK-3 β and p38 MAP kinase (MAPK11). These targets were chosen in order to examine, and quantify, three major signaling pathways activated via the EPOR. Both rHuEPO and peginesatide induced a dose dependent phosphorylation of all six signal transduction factors, but with different potencies (EC₅₀) and efficacies (i.e., maximal stimulation) (Figure 1 and Table 2). When potencies were compared, rHuEPO was approximately 50-fold more potent than peginesatide for 5 out of 6 mediators (Table 2). However, peginesatide was approximately 450-fold less potent than rHuEPO in stimulating the phosphorylation of p38 MAP kinase, a 9-fold larger difference than observed for other targets. When the efficacies (or upper plateaus) of phosphorylation after rHuEPO and peginesatide treatments were compared, they were found to be similar for STAT5, ERK1/2, GSK-3 β , and p38 MAP kinase (Table 2). In contrast, the maximal phosphorylation of AKT and STAT3 induced by peginesatide differed, and was ~85% and ~75% (respectively) of the levels induced by rHuEPO.

To evaluate whether the above concentration dependent features might reflect time points chosen for analyses, the time course of phosphorylation for these same molecules was evaluated. The data show that maximal phosphorylation of AKT, STAT5, STAT3, ERK1/2 and GSK-3 β occurred within 5-20 minutes after ligand addition, whereas p38 MAP kinase phosphorylation exhibited a more gradual increase over time (Figure 2). While rHuEPO- and peginesatide-mediated phosphorylation of STAT5, ERK1/2, GSK-3 β and p38 demonstrated comparable levels over time, AKT and STAT3 again showed at least modest differences in maximal stimulation.

Comparisons of gene expression events modulated by peginesatide and rHuEPO

In order to understand if the observed modest differences in signal transduction between rHuEPO and peginesatide might have further functional associations, we examined the effect of ESA treatment on gene expression. Specifically, UT-7/EPO cells were treated for 1.5 h with rHuEPO or peginesatide to induce immediate-early gene expression. Treatment with stem cell factor (SCF) or vehicle alone served as controls. Analyses of transcripts by microarray demonstrated that rHuEPO and peginesatide mediated very similar changes in mRNA expression when compared to untreated or SCF treated cells, and this was visually illustrated by principal component analysis (Figure 3A). Here, the relatedness of the global gene expression levels for each sample is reflected in its similarity to other treatments. Thus, biological replicates undergoing equal treatment cluster together in the 2D representation, while each treatment condition occupies its own position. Analyses demonstrate that peginesatide and rHuEPO treatments induce a similar gene induction profile whereas gene induction profiles induced not only by vehicle, but also SCF treatments, are distinct.

A more detailed analysis of modulated transcripts revealed that 32 genes were induced 2-fold in UT-7/EPO cells by peginesatide and by rHuEPO treatment. There were no genes induced by rHuEPO that were not induced by peginesatide, or vice versa. The modulated expression of these genes is illustrated in the heat map in Figure 3B. Quantification and specific probe information are provided in Table 3 (data expressed as fold change in comparison to vehicle treated cells). Only a subset of these genes was modulated by SCF (Table 3). Data from two separate probe sets, however, did consistently indicate that rHuEPO induces approximately 2-fold more Suppressor of Cytokine Signaling 3 (SOCS3) than does peginesatide.

Relative levels of modulation of the above genes were also independently verified by quantitative RT-PCR (Table 4). Genes shown were significantly induced by peginesatide and rHuEPO treatment compared to control treated cells ($p < 0.01$). In general, induction by peginesatide tended to be lower than for rHuEPO. A few genes that were identified as up-regulated in the microarray analysis were not confirmed to be up-regulated by at least 2 fold by RT-PCR, namely EPAS1, PLSCR1, PMP22, RAB11FIP1, RHOB, and VMP1.

Several of these identified genes have previously been shown to be induced by rHuEPO (Table 3). For example, SOCS3, a STAT5 response gene, is a known negative feedback regulator of EPOR signaling [26]. Additionally, this analysis now implicates SOCS1 and SOCS2 as part of the feedback loop. EGR1 and c-FOS are known targets of the MAP kinase pathway, and Prostaglandin E2, a product of PTGS2, has been reported to enhance EPOR signaling [27, 28] [29].

Oncostatin M (OSM), which is another STAT5 target gene, is important for maintaining erythroid and megakaryocytic progenitor cell pools since knockout of its receptor reduces circulating erythrocyte and platelet levels [30–33]. OSM also has recently been shown to increase hepcidin expression, a peptide that suppresses the release of iron from cellular stores, which is needed to support erythropoiesis [34]. Two other genes known to regulate iron metabolism by modulating the liver expression of hepcidin, namely BMP2 and GDF-15, were also found to be modulated by both peginesatide and rHuEPO [35–38].

In order to evaluate peginesatide and rHuEPO modulated genes in a larger biological context, gene set testing was conducted to identify activated molecular pathways. Pathways were considered up-regulated if the FDR corrected p value was < 0.05 , and at least 25% of the genes within the given pathway were modulated. We find that peginesatide and rHuEPO regulate similar EPOR-mediated response pathways consistent with their roles in driving erythropoiesis. The analysis identified pathways involved in erythroid differentiation, regulation of hematopoiesis by cytokines, cytokine networks, cell cycle and DNA replication, transcription, MAPK activation, and JAK/STAT cascade activation (Table 5).

Analysis of the increase in SOCS mRNA expression

The induction of several genes showed a significant difference between peginesatide and rHuEPO ($p < 0.01$), however, the most notable difference was in the expression observed for SOCS3 (Table 4). To determine if the results might reflect a sampling bias based on the length of treatment, we also performed a time course study using RT-PCR. These studies confirmed that at most time points most of the transcripts showed similar or slightly lower expression levels induced by peginesatide compared to rHuEPO (data not shown). SOCS3 consistently showed significantly lower induction in response to peginesatide versus rHuEPO, further pointing to this response as a significant difference in response to these EPOR ligands (Figure 4).

Since it has previously been reported that SOCS3 expression is dependent upon p38 MAPK activation [45–47], it was of interest to determine if the reduced phosphorylation of p38 after peginesatide vs. rHuEPO treatment plays a role in the lower level of SOCS3 induction. Therefore, cells were pre-treated with the p38 inhibitor SB202190 prior to rHuEPO treatment and RT-PCR analysis. It was found that the induction of SOCS3 was partially reduced (~20–30%) confirming that the reduced activation of p38 after peginesatide treatment contributes to the reduced expression of SOCS3 mRNA.

Western blotting analysis was used to determine whether the difference in SOCS3 mRNA expression leads to differences in SOCS3 protein levels. After overnight starvation, UT7/EPO cells were treated for 1–24 hours with peginesatide or rHuEPO. Only limited differences in SOCS3 protein levels were observed which were not statistically significant. This may relate to variable time-frames for protein expression (as compared to ligand-induced phosphorylation and transcription events); the half-life of pre-existing high levels of SOCS3 in UT-7/EPO cells; and/or additional SOCS3 regulatory circuits.

Discussion

The development of second and third generation ESAs has focused, in part, on products with properties that allow for dosing at 2 to 4-week intervals. Products such as darbepoetin and methoxy polyethylene glycol-epoetin beta, which are rHuEPO-based and rely on post-translational modifications to enhance plasma half life, might be expected to have similar receptor recognition, binding, and signaling events. However, evidence exists that the PEGylation of methoxy polyethylene glycol-epoetin beta impacts EPOR on-off rates with an unknown impact on signaling [39]. In contrast the synthetic peptide EMP1, which shares no sequence similarity with erythropoietin, binds the EPOR with distinct kinetics and results in a receptor-ligand structure with a conformation different from that induced by rHuEPO [3–5]. However, there has been limited characterization of these next generation products with respect to how changes in receptor engagement might impact signaling, and therefore function.

In the current study, global approaches were taken to compare signal transduction events activated by peginesatide vs. rHuEPO. Phosphoproteomics, (using two different proteases to generate phosphopeptides) identified a significant number of known and novel phosphorylation events in response to ESA treatment. The complete overlap in phosphopeptides identified after rHuEPO or peginesatide treatment indicates that these two EPOR ligands activate very similar signaling pathways. These first include known JAK/STAT, PI3 kinase, MAP kinase, PLC γ and VAV1 pathways that are mediated through SHIP1, SHP2, CBL, CrkL, GAB and GRB2 [11, 13–16, 18, 21, 22, 40]. In the JAK/STAT pathway we confirmed the activation of STAT3 and STAT5, which are thought to support proliferation and suppress differentiation induced by rHuEPO [23, 25]. In addition to ERK1/2 and p38 within the MAP kinase pathway, ERK5 was unexpectedly activated. By examining other detected phosphopeptides that did not change in intensity with treatment, certain previously identified rHuEPO-induced phosphorylation events were not observed in UT-7/EPO cells, such as Lyn and Btk [41–43]. In rodent cell lines or erythroblasts, Lyn and Btk have been shown to play a role in regulating erythropoietic proliferation and differentiation, but there is little data on their role in human cells.

To directly compare the induction of cell signaling between peginesatide and rHuEPO, three major signaling pathways in UT-7/EPO cells, namely the JAK/STAT (STAT3, STAT5), MAP kinase (ERK1/2, p38) and PI3 kinase (AKT, GSK-3 β) pathways, were examined using a robust quantitative method. Interestingly, this analysis unexpectedly revealed that rHuEPO itself activated these pathways with potencies (EC₅₀s) that differed over a 120-fold

concentration range. Given that the rHuEPO potency for proliferation of UT-7/EPO cells is ~ 35 pM [6], this suggests that proliferation may involve different levels of activation of different pathway components. Peginesatide treatment also induced a similar pattern of EC₅₀ values at approximately a 50-fold higher threshold (except for p38 which was 450-fold higher). Thus, the JAK/STAT and MAP kinase pathways are significantly activated consistent with a role in proliferation. The PI3 kinase survival pathway is somewhat less activated, though it is unclear if this reflects a lesser requirement for full activation of the individual components measured. In contrast, the lesser activation of STAT3 would indicate that this molecule plays a less significant role for proliferation in UT-7/EPO cells, differing from previous suggestions in UT-7 cells, but matching observations in vivo in mice [23, 44]. To our knowledge, this is the first report to quantitatively evaluate the impact of EPOR engagement on multiple signaling pathways simultaneously, and it will be interesting to determine if the finding of differential thresholds for the activation of different pathways is replicated for other ligand and receptor systems.

To evaluate whether kinetics and signaling differences observed for rHuEPO compared to peginesatide relate to downstream consequences, we profiled genes induced by peginesatide and rHuEPO. We found that both ESAs induced the same set of transcripts in UT-7/EPO cells, which could be easily distinguished from those induced by SCF.

Quantitative RT-PCR comparison of the level of induction of these genes suggested that in general peginesatide induced gene expression somewhat less than rHuEPO, although this only reached statistical significance for 8 genes. The most substantial difference between peginesatide and rHuEPO expression is in the induction of SOCS3 mRNA, with peginesatide treatment leading to 2- to 3- fold lower levels. SOCS3 mRNA expression has been reported to be dependent on STAT3/5 and p38 MAP kinase activation [45–47]. We also find that p38 MAP kinase activation contributes to SOCS3 mRNA expression. Therefore, the lower stimulation of p38 MAP kinase by peginesatide partially leads to a lower induction of SOCS3 mRNA.

However, we do not find that SOCS3 protein levels are significantly modulated in UT-7/EPO cells. SOCS proteins are general suppressors of cytokine signaling via multiple mechanisms, including for the cytokines, granulocyte colony stimulating factor and IL-6. For SOCS3, and within an EPO receptor/JAK2 complex context, substantial regulation beyond transcriptional induction occurs. Specifically, SOCS3 is a component of a multimeric E3 ubiquitin ligase complex whose activation depends upon EPOR docking at EPOR/JAK2 complexes, involves competition for EPOR PY344 sites by CIS, and likely depends upon EPOR and/or JAK2 phosphorylation events [26, 48–51]. Additionally, the actions of deubiquitinating enzymes and other SOCS factors can cross-regulate (e.g., SOCS-2 and -3) [26] [52]. In our recent analyses of rHuEPO-regulated transcriptomes in developing primary CFUe, pro-erythroblasts, and erythroblasts, we have discovered that one major set of rHuEPO-response genes that differs substantially among developmental stages comprises negative-feedback factors (including SOCS factors) (manuscript in preparation). Future studies are planned to better understand SOCS3 regulation after EPOR activation, and subsequently within peginesatide vs. rHuEPO activation contexts.

In conclusion, a global comparison of signaling induced by peginesatide and rHuEPO in UT-7/EPO cells suggests that they activate very similar pathways, consistent with their roles in stimulating the proliferation and maturation of erythroid progenitor cells to drive RBC production.

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Figure 1.

Peginesatide and rHuEPO potently induce the phosphorylation of AKT, GSK-3 β , STAT5, STAT3, AKT1, ERK1/2, and p38 α . Lysates were prepared from UT-7/EPO cells treated with a dose response of rHuEPO (○, 0.01 – 10 nM) or peginesatide (■, 0.1 – 50 nM) for 10 minutes. Clarified lysates were analyzed for protein phosphorylation using MesoScale Discovery multi-spot signaling plates pre-coated with the appropriate capture antibodies. For AKT, GSK-3 β , ERK1/2, and p38, percent phosphorylation was calculated by determining the ratio of phosphorylated protein to total protein detected in each well. For STAT5 and STAT3, relative phosphorylation was calculated based upon maximum signal. Data are representative of at least 3 independent experiments.

Figure 2.

Time course analysis of rHuEPO and peginesatide induced signal transduction. Lysates were prepared from UT-7/EPO cells treated for 0 to 60 minutes with 10 nM rHuEPO (○) or 100 nM peginesatide (■). These concentrations were chosen to be 10 fold above the amount needed to induce maximal phosphorylation. Percent AKT, GSK-3 β , STAT5, STAT3, ERK1/2, and p38 phosphorylation was determined using Meso Scale Discovery multi spot plates as in Figure 1.

A.

B.

Figure 3.

Global gene modulation evaluation using principal component analysis. A. UT-7/EPO cells were treated for 90 minutes with vehicle, 5 U/mL rHuEPO, 20 nM peginesatide, or 10 nM SCF. Principal component analysis software was utilized to capture the relatedness of gene modulation induced by various treatments. This is displayed in terms of principal components, allowing for dimensionality reduction of the data from ~55,000 probe sets to the most informative components. Three biological replicates for each treatment are shown. B. Heat map of genes significantly regulated by peginesatide and rHuEPO, with white representing basal expression and progressing to red representing gene induction.

Figure 4.

A. RT-PCR time course analysis of SOCS3 gene expression. UT-7/EPO cells were stimulated with 5 U/mL rHuEPO or 20 nM peginesatide for 0 – 4.5 hours. mRNA was isolated and subjected to RT-PCR analysis using primers specific for SOCS3. SOCS3 gene expression was normalized to the endogenous control gene HPRT1. *Difference in SOCS3 induction is significant for each time point ($p < 0.0006$). B. UT-7/EPO cells were preincubated with the p38 inhibitor SB202190 for 1 hour before stimulation with rHuEPO for 1 hour. RT-PCR analysis was conducted as above. *Difference in SOCS3 induction with p38 inhibition is significant ($p = 0.003$).

Table 1
Summary of proteins modulated in tyrosine phosphorylation in response to peginesatide or rHuEPO treatment.

Gene Symbol	Site	Protein Name	Fold-Change over Control			
			Trypsin Digest		GluC Digest	
			rHuEPO	Peginesatide	rHuEPO	Peginesatide
<i>Protein kinase, Tyr (non-receptor)</i>						
FRK	§497	Tyrosine-protein kinase FRK			6.4	4.7
JAK2	§570	Tyrosine-protein kinase JAK2	4.4	6.0	15.7	9.8
JAK2	§813	Tyrosine-protein kinase JAK2	11.3	6.2		
JAK2	§1007	Tyrosine-protein kinase JAK2	34.8	14.4		
JAK2	§1007, §1008	Tyrosine-protein kinase JAK2	5.5	3.4	90.2	20.4
<i>Kinase (non-protein)</i>						
PIK3CA	§508	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit			40.4	9.1
PIK3R1	§607	Phosphatidylinositol 3-kinase regulatory subunit alpha			4.9	2.9
<i>Phosphatase</i>						
SHIP1	§865	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1			3.2	2.5
SHIP1	§915	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1	61.7	46.1		
SHIP2	§986	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2	31.2	36.4	77.2	68.6
SHIP2	§1135	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2	9.1	3.3	32.9	12.7
SHP-2	§584	Tyrosine-protein phosphatase non-receptor type 11	33.6	16.8	185.7	64.0
PTPN18	§387	Tyrosine-protein phosphatase non-receptor type 18			98.0	20.0
<i>Transcriptional regulator</i>						
KLF5	95	Krüppel-like factor 5			121.7	21.8
STAT1	§701	Signal transducer and activator of transcription 1-αβ			88.6	5.2
STAT3	§704	Signal transducer and activator of transcription 3	12.8	5.5		

Gene Symbol	Site	Protein Name	Fold-Change over Control			
			Trypsin Digest		GluC Digest	
			rHuEPO	Peginesatide	rHuEPO	Peginesatide
STAT3	\$705	Signal transducer and activator of transcription 3	14.1	5.6	18.8	3.7
STAT5A	\$694	Signal transducer and activator of transcription 5A	36.7	28.2	139.2	88.3
STAT5B	682	Signal transducer and activator of transcription 5B			107.5	86.1
STAT5B	\$699	Signal transducer and activator of transcription 5B			103.4	75.2
<i>Adaptor/scaffold</i>						
CBL	\$700	E3 ubiquitin-protein ligase CBL	8.4	8.1		
CRKL	\$127	Crk-like protein	9.6	10.0		
CD2AP	\$361	CD2-associated protein			253.2	58.0
FRS2	\$195	Fibroblast growth factor receptor substrate 2			6.6	4.3
GAB1	183	GRB2-associated-binding protein 1			32.4	18.6
GAB1	\$285	GRB2-associated-binding protein 1			5.1	6.5
GAB1	\$317	GRB2-associated-binding protein 1			12.9	14.2
GAB1	\$627	GRB2-associated-binding protein 1	3.9	10.6		
GAB1	\$659	GRB2-associated-binding protein 1	47.3	84.6		
GAB3	183	GRB2-associated-binding protein 3			18.0	9.2
GAB3	\$560	GRB2-associated-binding protein 3			7.8	7.2
GRB2	52	Growth factor receptor-bound protein 2			20.1	4.9
HGS	\$329, \$334	Hepatocyte growth factor-regulated tyrosine kinase substrate			3.3	3.4
SHC1	\$349	SHC-transforming protein 1	6.8	10.3		
SHC1	\$427	SHC-transforming protein 1	9.3	14.7		
SH2B3	572	SH2B adapter protein 3			115.8	49.9
SPRY4	\$52	Protein sprouty homolog 4			8.8	4.6
<i>Adhesion or extracellular matrix protein</i>						
CD33	\$340	Myeloid cell surface antigen CD33			13.3	3.4

Gene Symbol	Site	Protein Name	Fold-Change over Control			
			Trypsin Digest		GluC Digest	
			rHuEPO	Peginesatide	rHuEPO	Peginesatide
MLLT4	\$1480	Afadin			55.8	9.6
PCDHAC2	521, 532, 539	Protocadherin alpha-C2			7.9	3.4
<i>Cell cycle regulation</i>						
SEPT2	\$17	Septin-2	13.6	11.0		
<i>Chromatin, DNA-bionding, DNA repair or DNA replication protein</i>						
ERCC6	882, 901	DNA excision repair protein ERCC-6			52.7	45.8
TOP2B	928	DNA topoisomerase 2-beta			3.5	5.3
<i>Cytoskeletal protein</i>						
ADD2	440	Beta-adducin	8.4	8.3		
EZR	499	Ezrin			31.1	16.4
SPTA1	\$2332	Spectrin alpha chain, erythrocyte			24.1	4.6
SPTB	15	Spectrin beta chain, erythrocyte			18.9	5.9
<i>Enzyme, misc.</i>						
ALDOA	\$363	Fructose-bisphosphate aldolase A			4.4	3.3
OTC	55	Ornithine carbamoyltransferase, mitochondrial	24.9	31.1		
PLCG1	\$775	1-phosphatidylinositol-4,5-bisphosphate phosphodiesteraseγ1			5.0	3.4
PLCG1	\$783	1-phosphatidylinositol-4,5-bisphosphate phosphodiesteraseγ1	12.1	12.6	50.6	31.0
PLCG1	833	1-phosphatidylinositol-4,5-bisphosphate phosphodiesteraseγ1			27.4	6.8
<i>G protein or regulator</i>						
RICS	752	Rho/Cdc42/Rac GTPase-activating protein RICS	9.1	4.2		
VAV1	603	Vav	6.2	6.6		
VAV1	\$844	Vav	16.8	7.6	5.4	3.7
<i>Protein kinase, Ser/Thr (non-receptor)</i>						

Gene Symbol	Site	Protein Name	Fold-Change over Control			
			Trypsin Digest		GluC Digest	
			rHuEPO	Peginesatide	rHuEPO	Peginesatide
MAPK1	\$186	Mitogen-activated protein kinase 1 (ERK2)	29.4	46.2		
MAPK3	\$204	Mitogen-activated protein kinase 3 (ERK1)	24.5	34.9		
MAPK7	\$220	Mitogen-activated protein kinase 7 (ERK5)	15.7	24.3		
MAPK11	\$182	Mitogen-activated protein kinase 11 (p38beta)			2.8	3.1
SgK269	\$635	Protein containing a protein kinase domain	10.3	8.2		
<i>Receptor, channel, transporter or cell surface protein</i>						
EPOR	\$504	Erythropoietin receptor			18.4	12.2
FCGR2A	\$288	Low affinity immunoglobulin gamma Fc region receptor II-a			17.2	9.3
FCGR2A	\$304	Low affinity immunoglobulin gamma Fc region receptor II-a	2.6	2.7		
SLC4A7	\$44	Sodium bicarbonate cotransporter 3			12.7	3.7
SURF1	274, 279, 287	Surfeit 1	9.4	8.1		
TNFRSF19	378	Tumor necrosis factor receptor superfamily member 19			2.9	2.5
<i>Ubiquitin conjugating system</i>						
FBXW11	537, 539	F-box/WD repeat-containing protein 11			98.2	11.3

Red text denotes proteins previously reported to be phosphorylated in response to rHuEPO.

A \$ symbol denotes a phosphorylation site previously reported in the literature.

Table 2

Quantitative comparison of peginesatide and rHuEPO signaling

Substrate	rHuEPO Potency (EC ₅₀ , pM)	Peginesatide Potency (EC ₅₀ , pM)	Potency Ratio (rHuEPO/Peginesatide)	Peginesatide Efficacy (% Max Activation)
phospho-AKT	90	2230	42 ± 22	85 ± 9
phospho-GSK-3β	21	745	42 ± 6	96 ± 3
phospho-STAT5	9	597	62 ± 6	101 ± 3
phospho-STAT3	369	11480	54 ± 22	77 ± 13
phospho-ERK1/2	7	354	54 ± 34	92 ± 8
phospho-p38	3	1508	462 ± 36	98 ± 7

Table 3

Summary of gene expression analysis

Gene Symbol	Gene Title	UniGene ID	ProbeSet Name	pegisatide (Fold Change)	rHuEPO (Fold Change)	SCF (Fold Change)	Overall p-value (ANOVA)	p-value (pegisatide vs. rHuEPO)
AREG	amphiregulin (schwannoma-derived growth factor)	Hs.270833	205239_at	4.8	4.9	2.3	5.7 E-05	9.1 E-01
BMP2	bone morphogenetic protein 2	Hs.73853	205289_at	5.7	7.0	1.3	5.2 E-08	1.1 E-02
BMP2	bone morphogenetic protein 2	Hs.73853	205290_s_at	2.9	4.0	1.1	2.3 E-05	6.1 E-02
CCL2	chemokine (C-C motif) ligand 2	Hs.303649	216598_s_at	33.3	36.4	8.1	1.1 E-06	6.9 E-01
CD69	CD69 antigen (p60, early T-cell activation antigen)	Hs.208854	209795_at	16.3	17.9	5.3	4.6 E-09	4.8 E-01
CD80	CD80 antigen (CD28 antigen ligand 1, B7-1 antigen)	Hs.838	1555689_at	6.0	6.0	1.8	5.3 E-05	9.9 E-01
CISH	cytokine inducible SH2-containing protein	Hs.8257	223961_s_at	10.9	12.9	1.1	8.1 E-07	2.1 E-02
CISH	cytokine inducible SH2-containing protein	Hs.8257	223377_x_at	5.3	6.5	1.2	8.1 E-08	5.5 E-02
CISH	cytokine inducible SH2-containing protein	Hs.8257	221223_x_at	4.3	5.4	1.1	2.9 E-06	1.9 E-02
CXCL2	chemokine (C-X-C motif) ligand 2	Hs.75765	209774_x_at	4.0	4.1	1.5	5.6 E-05	7.2 E-01
DUSP2	dual specificity phosphatase 2	Hs.1183	204794_at	6.3	6.0	4.3	1.0 E-06	6.1 E-01
EGR1	early growth response 1	Hs.326035	201693_s_at	12.2	13.7	7.1	9.3 E-08	2.0 E-01
EGR1	Early growth response 1	Hs.326035	227404_s_at	10.4	9.7	7.8	5.7 E-09	4.9 E-01
EGR1	early growth response 1	Hs.326035	201694_s_at	7.1	7.5	5.5	8.4 E-09	5.0 E-01
EGR2	early growth response 2 (Krox-20 homolog, Drosophila)	Hs.1395	205249_at	7.9	9.5	3.2	2.9 E-07	4.9 E-02
EGR3	early growth response 3	Hs.534313	206115_at	11.6	11.8	8.5	3.7 E-07	8.1 E-01
EGR4	early growth response 4	Hs.549031	207768_at	11.2	12.0	8.6	3.2 E-08	2.0 E-01
EGR4	early growth response 4	Hs.549031	207767_s_at	3.9	3.5	3.2	1.3 E-04	5.1 E-01
EPAS1	Endothelial PAS domain protein 1	Hs.468410	242868_at	4.2	5.6	1.6	8.5 E-06	1.0 E-01
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	Hs.25647	209189_at	4.2	4.6	2.1	2.2 E-05	5.4 E-01
GDF15	growth differentiation factor 15	Hs.515258	221577_x_at	5.2	5.1	2.6	4.4 E-08	9.2 E-01
IER3	immediate early response 3	Hs.76095	201631_s_at	13.3	16.6	5.8	2.3 E-10	1.3 E-02
IRF1	interferon regulatory factor 1	Hs.436061	202531_at	2.9	4.9	1.2	1.2 E-07	5.4 E-04
KLF6	Kruppel-like factor 6	Hs.285313	208961_s_at	5.7	7.5	1.3	5.2 E-09	1.3 E-03
KLF6	Kruppel-like factor 6	Hs.285313	208960_s_at	5.7	6.9	1.3	1.9 E-06	5.4 E-02

Gene Symbol	Gene Title	UniGene ID	ProbeSet Name	pegnesatide (Fold Change)	rHuEPO (Fold Change)	SCF (Fold Change)	Overall p-value (ANOVA)	p-value (pegnesatide vs. rHuEPO)
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	Hs.517617	36711_at	4.6	5.1	2.5	1.2 E-09	8.3 E-02
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Hs.319171	223218_s_at	2.0	2.4	1.1	6.7 E-06	1.2 E-01
OSM	oncostatin M	Hs.248156	214637_at	46.1	54.5	1.1	2.5 E-08	2.6 E-01
OSM	oncostatin M	Hs.248156	230170_at	36.6	50.2	1.2	1.9 E-08	4.2 E-03
PHLDA1	pleckstrin homology-like domain, family A, member 1	Hs.549112	217997_at	8.8	10.1	3.6	5.6 E-09	2.4 E-01
PHLDA1	pleckstrin homology-like domain, family A, member 1	Hs.549112	217996_at	7.8	7.7	3.4	1.1 E-07	8.8 E-01
PLSCR1	Phospholipid scramblase 1	Hs.130759	241916_at	2.7	3.4	1.2	2.3 E-06	2.7 E-02
PMP22	Peripheral myelin protein 22	Hs.372031	1565638_at	3.7	4.6	1.2	1.3 E-05	2.2 E-01
PMP22	Peripheral myelin protein 22	Hs.372031	1565639_a_at	3.5	4.5	1.2	2.8 E-08	2.1 E-02
PMP22	Peripheral myelin protein 22	Hs.372031	243050_at	2.0	2.8	1.1	9.6 E-05	4.1 E-03
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Hs.196384	204748_at	13.5	16.0	4.9	1.8 E-07	2.6 E-01
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Hs.196384	1554997_a_at	7.9	6.9	2.9	1.9 E-07	3.3 E-01
RAB11FIP1	RAB11 family interacting protein 1 (class I)	Hs.191179	219681_s_at	4.0	4.3	1.4	1.3 E-07	4.8 E-01
RAB11FIP1	RAB11 family interacting protein 1 (class I)	Hs.191179	225177_at	3.4	3.6	1.2	5.1 E-06	3.4 E-01
RHOB	ras homolog gene family, member B	Hs.502876	212099_at	3.1	3.1	2.0	3.9 E-05	9.6 E-01
SOCS1	suppressor of cytokine signaling 1	Hs.506640	210001_s_at	1.9	2.2	0.9	2.1 E-04	
SOCS2	suppressor of cytokine signaling 2	Hs.485572	203372_s_at	3.9	3.5	1.2	9.3 E-05	4.6 E-01
SOCS2	suppressor of cytokine signaling 2	Hs.485572	203373_at	2.7	3.1	1.2	1.6 E-07	1.9 E-01
SOCS3	suppressor of cytokine signaling 3	Hs.527973	227697_at	35.1	74.8	0.7	8.9 E-10	7.6 E-04
SOCS3	suppressor of cytokine signaling 3	Hs.527973	206359_at	22.0	41.4	1.2	7.3 E-07	4.0 E-02
THBS1	Thrombospondin 1	Hs.164226	235086_at	2.6	2.8	1.8	1.4 E-06	1.1 E-03
VMP1	likely ortholog of rat vacuole membrane protein 1	Hs.444569	1569003_at	11.2	11.1	2.2	6.0 E-06	9.6 E-01

Table 4

Quantification of peginesatide- and rHuEPO-induced gene induction

RT-PCR Fold-induction (90 minutes)			
Gene Symbol	Peginesatide	rHuEPO	p < 0.01
AREG	2.7	2.6	
BMP2	4.9	5.9	
CCL2	9.8	10.0	
CD69	9.4	11.7	
CD80	2.3	2.7	
CISH	15.9	26.5	
CXCL2	4.6	7.0	*
DUSP2	3.9	3.7	
EGR1	42.4	63.6	*
EGR2	6.9	8.5	
EGR3	20.0	22.5	
EGR4	6.8	7.8	
FOS	5.9	11.8	*
IER3	4.4	5.9	*
IRF1	3.4	6.3	*
KLF6	3.7	4.6	
MAFF	3.0	2.5	
MYC	2.9	2.8	
NFKBIZ	2.1	2.7	
OSM	100.4	128.8	
PHLDA1	3.6	4.1	
PTGS2	3.2	4.5	*
SOCS1	4.2	5.5	
SOCS3	26.8	77.9	*
THBS1	2.3	3.0	*

* denotes a significant difference ($p < 0.01$) in the magnitude of rHuEPO vs. peginesatide gene induction

Table 5

Peginesatide and rHuEPO modulated molecular pathways

Up-Regulated Molecular Pathways	Brief Description of Pathway
BIOCARTA_ERYTH_PATHWAY	Erythrocyte Differentiation
BIOCARTA_STEM_PATHWAY	Regulation of hematopoiesis by cytokines
BIOCARTA_CYTOKINE_PATHWAY	Cytokine network
ST_STAT3_PATHWAY	JAK/STAT cascade
BIOCARTA_ARENRF2_PATHWAY	Transcriptional regulation
BIOCARTA_CCR5_PATHWAY	Cytokine network
BIOCARTA_CD40_PATHWAY	Transcriptional regulation of NFκB
BIOCARTA_G2_PATHWAY	Cell Cycle: G2/M Checkpoint

FDR corrected $p < 0.05$, percent contributing genes $> 25\%$