Eirini Kostopoulou*, Andrea Paola Rojas-Gil, Alexia Karvela and Bessie E. Spiliotis*

Epidermal growth factor receptor (EGFR) involvement in successful growth hormone (GH) signaling in GH transduction defect

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Abstract

Background: Growth hormone (GH) transduction defect (GHTD) is a growth disorder with impaired signal transducer and activator of transcription 3 (STAT3) phosphorylation mediated by overexpression of cytokine-inducible SH2-containing protein (CIS), which causes increased growth hormone receptor (GHR) degradation. This study investigated the role of epidermal growth factor (EGF) in the restoration of normal GH signaling in GHTD.

Methods: Protein expression, cellular localization and physical contact of proteins of the GH and EGF signaling pathways were studied by Western immunoblotting, immunofluorescence and co-immunoprecipitation, respectively. These were performed in fibroblasts of one GHTD patient (P) and one control child (C) at the basal state and after induction with human GH (hGH) 200 μg/L (GH200), either with or without silencing of CIS mRNA, and after induction with hGH 1000 μg/L (GH1000) or 50 ng/mL EGF.

Results: The membrane availability of the EGF receptor (EGFR) and the activated EGFR (pEGFR) was increased in P only after simultaneous GH200 and silencing of CIS mRNA or with GH1000, whereas this occurred in C after GH200 alone. After EGF induction, the membrane localization of GHR, STAT3 and that of EGFR were increased in P more than in C.

Conclusions: In conclusion, in GHTD, the EGFR seems to participate in successful GH signaling, but induction of GHTD fibroblasts with a higher dose of hGH is needed. The EGF/EGFR pathway, in contrast to the GH/GHR pathway, seems to function normally in P and is more primed compared to C. The involvement of the EGFR in successful GH signaling may explain the catch-up growth seen in the Ps when exogenous hGH is administered.

Keywords: cytokine-inducible SH2-containing protein (CIS); epidermal growth factor receptor (EGFR); GH pathway; growth hormone; growth hormone transduction defect (GHTD); phosphorylated STAT3 (pSTAT3).

Introduction

Growth hormone (GH) is a key regulator of body growth and metabolism via its effects on cellular function and differentiation [1, 2]. Specifically, it plays a major role in the metabolism of proteins, lipids and carbohydrates and in bone turnover, immune function and DNA synthesis, as the transcription of the specific genes required for these processes is regulated by GH [3].

When GH binds to its receptor (GHR), the receptor-associated Janus kinase 2 (JAK2) is activated and tyrosyl phosphorylation is initiated of both JAK2 and the GHR. Several signaling cascades are then induced, including the JAK/signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK) and phosphatidyloinositol 3 kinase (PI3K) pathways. The JAK/STAT pathway is the principal signaling mechanism for cell proliferation, differentiation, migration and apoptosis, primarily through the GH/insulin growth factor 1 (IGF1) pathway [4, 5].

Due to its significant actions, cellular responsiveness to the GH is strictly regulated. Soon after the transduction of the GH signal begins, regulatory mechanisms are activated. The negative regulation of the GHR involves several mechanisms through which excessive cellular growth is limited. Factors that are involved in the negative regulation of GH-induced JAK/STAT signaling are the following: the protein tyrosine phosphatases, the protein inhibitors

***Corresponding authors: Eirini Kostopoulou,** MD, PhD, and **Bessie E. Spiliotis,** MD, Pediatric Endocrine Research Laboratory, Division of Pediatric Endocrinology and Diabetes, Department of Pediatrics, University of Patras School of Medicine, 26504, Rio, Patras, Greece, Phone: +30 6972070117 (E. Kostopoulou); +30 6977000947 (B.E. Spiliotis),
E-mail: eirini.kost@gmail.com (E. Kostopoulou); besspil@endo.gr (B.E. Spiliotis)
Andrea Paola Rojas-Gil: Faculty of Human Movement and Quality of Life Sciences, Department of Nursing, University of Peloponnese, Sparta, Lakonias, Greece
Alexia Karvela: Pediatric Endocrine Research Laboratory, Division of Pediatric Endocrinology and Diabetes, Department of Pediatrics, University of Patras School of Medicine, Patras, Greece

of activated STATs (PIAS) and the suppressors of cytokine signaling (SOCS) proteins, consisting of eight intracellular proteins, SOCS 1–7 and the cytokine-induced Src homology-2 protein (CIS) [6, 7]. CIS, an E3 ubiquitin (Ub) ligase, inhibits GHR signaling by competing with STAT5b for common GHR phosphotyrosine-binding sites and by a proteasome-dependent mechanism that involves GHR internalization and degradation using a ubiquitination system [8–10].

Another pathway that is significant for normal cell growth is the epidermal growth factor (EGF) signaling pathway, which crosstalks with the GH signaling pathway. EGF stimulates cell growth, proliferation and differentiation through its receptor, EGFR. Crosstalk between the GH and EGF signaling pathways has been previously described, in which the GH stimulates tyrosyl phosphorylation of the EGFR, which is independent of the receptor's intrinsic tyrosine-kinase activity [11, 12]. Furthermore, in 3T3-F442A pre-adipocytes, the GH has been found to stimulate threonine phosphorylation of the EGFR [13].

Several defects of the GH pathway resulting in IGF-1 deficiency and severe growth retardation have been described, which cause the GH insensitivity syndrome (GHIS), such as the Laron syndrome, STAT5b deficiency, primary acid-labile subunit (ALS) deficiency, IGF-1 mutations and IGF-1 receptors mutations [14–17]. Biosynthetic human growth hormone (hGH) therapy is unsuccessful in GHIS, which can only be treated with biosynthetic IGF-1 therapy [18]. Our group has described an additional GH/IGF-1 pathway disorder, the GH transduction defect (GHTD). GHTD is characterized by severe growth retardation with normal provoked and spontaneous GH secretion, abnormally low IGF-1 concentrations, significantly increased IGF-1 concentrations after induction with hGH during the IGF-1 generation test and significant "catch-up" growth after hGH therapy [19]. We have also reported that children with GHTD have increased GHR degradation due to overexpression of CIS and ubiquitinated CIS (uCIS) [20]. In an attempt to explain the observation that children with GHTD can overcome the GH signaling defect with the administration of hGH, we examined the possible involvement of the EGFR in this process.

Materials and methods

The study included one pre-pubertal GHTD child (P), [8 years and 6 months old; height standard deviation score (SDS), – 2.42 and bone age retardation SDS, > – 3.0] and one pre-pubertal age-matched control child (C) with normal stature. Both the children were recruited from the Outpatient Clinic of the Division of Pediatric Endocrinology and Diabetes of the University Hospital of Patras, Greece. The study was approved by the Ethics Committee of the University Hospital of Patras, Greece. After informed parental consent, the child with GHTD was treated with hGH and reached an adult height of 174 cm (– 0.44 SDS).

Cell cultures

Fibroblast cultures were established from the gingival biopsies obtained from P and C after informed parental consent and children's assent. The tissue pieces (0.5–1.0 mm^3; 2–3 pieces per dish) were plated onto 60-mm^2 culture dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine (Gibco, Invitrogen, Life technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS, Gibco, Invitrogen, Life technologies, Carlsbad, CA, USA), 50 IU/mL penicillin and 50 μg/mL streptomycin (Gibco, Invitrogen, Life technologies, Carlsbad, CA, USA), and incubated at 37 °C in 5% CO_2 atmosphere. The cultures were replenished with fresh medium every 3 days and then subcultivated in a 1 : 3 split ratio upon reaching confluency by using a trypsin/EDTA solution (Gibco, Invitrogen, Life technologies, Carlsbad, CA, USA). All experiments were carried out with the cultured fibroblasts between the second and sixth passages.

GH and EGF inductions

For the GH and EGF signal transduction pathway activation experiments, the P and C fibroblasts were cultured at 80% confluency, washed twice with phosphate-buffered saline (PBS), maintained for a 24-h period in serum-free DMEM and then treated with two different concentrations of hGH (Humatrope, Lilly, Indianapolis, IN, USA): 200 μg/L (GH200) and 1000 μg/L (GH1000) or with EGF (human recombinant EGF, Millipore, Billerica, MA, USA) 50 ng/mL.

Silence CIS (short interference RNA CIS/siRNA CIS)

The cellular localization of EGFR as well as the protein expression and the cellular localization of phosphorylated EGFR (pEGFR) were studied at baseline and after induction with GH200, either with or without siRNA CIS.

Silence CIS (siRNA) (Santa Cruz Biotechnology Inc, Dallas, TX, USA) was performed according to the manufacturer's instructions. A total of 2×10^5 cells/well were cultured in 2-mL antibiotic-free DMEM supplemented with FBS until the cells were 60%–80% confluent. For each transfection, 1 μg of siRNA was diluted in 100 μL siRNA Transfection Medium (sc-36868), solution A, and 6 μL of siRNA Transfection Reagent (sc-29528) in 100 μL siRNA Transfection Medium (sc-36868), solution B. Solution A was added to solution B and the mixture was incubated for 45 min at room temperature (RT).

The fibroblasts were washed once with 2 mL of siRNA Transfection Medium (sc-36868). The medium was aspirated. For each transfection, 0.8 mL of siRNA Transfection Medium was added to each tube containing solution A and solution B and the mixture was applied onto the washed cells. The cells were then incubated for 7 h at 37 °C in a CO_2 incubator. Subsequently, 1 mL of normal growth

medium containing 2 times the normal serum and antibiotic concentration was added to the transfection mixture and the cells were incubated for an additional 24 h. The medium was then aspirated and replaced with fresh 1×DMEM for 24 h. The cells were lysed for Western immunoblotting.

Western immunoblotting

The protein expression of CIS and its ubiquitinated isoform (uCIS), STAT3, phosphorylated STAT3 (pSTAT3), EGFR, pEGFR and GHR was studied by Western immunoblotting as follows: (a) at the basal state and after induction of the children's fibroblasts with 200 ng/mL hGH, either with or without silencing of CIS mRNA and (b) at the basal state and after induction of the fibroblasts for 15 min with GH200, GH1000 or EGF.

The following antibodies were used at the indicated concentrations: CIS (Santa Cruz Biotechnology Inc. Dallas, TX, USA, 1 : 250–500), STAT3 (Santa Cruz Biotechnology Inc., Dallas, TX, USA, 1 : 500), pSTAT3 (Cell Signalling Technology Inc., Danvers, MA, USA, 1 : 250), EGFR (Merck Millipore, MA, USA, 1 : 500), pEGFR (Santa Cruz Biotechnology Inc., Dallas, TX, USA, 1:200), GHR (Novus Biologicals, Littleton, CO, USA, 1:500) and β-tubulin (1:500), (Merck Millipore, Billerica, MA, USA, 1:500).

The cell lysates were diluted in homemade sodium dodecyl sulfate (SDS) sample buffer that was supplemented with β-merkaptoethanol (Sigma Aldrich, St Louis, MO, USA) and denatured. The mean protein concentration obtained by the used protein extraction method, before SDS was added, was 1 μg/μL (50 μg/50 μL) of the lysate. Coomassie blue staining of the gel was used as a loading control. Then, the cell lysates were loaded into wells in an SDS-polyacrylamide gel (10%: pSTAT3, pEGFR, EGFR, GHR and 12%: CIS). Fifty microliters of each sample was loaded into each lane. A prestained standard (Benchmark Prestained Protein Ladder, Invitrogen, Life technologies, Carlsbad, CA, USA) was used as a molecular weight marker. Voltage was applied along the gel and the proteins were separated depending on their size. Following the electrophoresis, the proteins were transferred to a nitrocellulose transfer membrane (Whatman, Protran, Sigma Aldrich, St. Louis, MO, USA), which was then incubated with one of the primary antibodies for 12 h and the appropriate second antibody for an additional 1 h. Immunoreactive proteins were visualized by chemiluminescence after the membranes were exposed to enhanced chemiluminescence (ECL) (H_2O_2 and luminal, GE Healthcare Biosciences AB, Sweden) and developed on photographic films. Autoradiograms were scanned and the bands corresponding to each protein were analyzed by densitometry. The protein expression of each protein was compared with the protein expression of β-tubulin, by using the program Scion image (version 4.0.3.2; Scion Corporation).

The Western blots were repeated several times. In our cell model, clear bands were not obtained, but the densitometry image analysis program used gave us results that showed consistent reproducibility and no major variability.

Immunofluorescence

The cellular localization of STAT3, pSTAT3, CIS, GHR, EGFR, pEGFR and Ub was studied by immunofluoresence. For the first part of the experiments, cells at the basal state or after induction with 200 μg/L were treated either with or without siRNA CIS. In the second part of the experiments, after induction of the cells with hGH (200 μg/L or 1000 μg/L) or EGF (50 ng/mL) or without induction, the cells were washed twice with PBS. The cells were fixed and permeabilized. The fixed cells were blocked for 1 h with 1% bovine serum albumin (BSA). The primary antibodies were applied at an appropriate dilution, time and temperature (pSTAT3, GHR: 1 : 20, EGFR, pEGFR, STAT3, CIS, Ub: 1 : 50, overnight at 4 °C, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After the incubation with the primary antibody, the cover slips were washed three times with 1% BSA followed by a 1-h incubation period with the secondary antibodies (FITC anti-rabbit, anti-mouse and anti-goat 1 : 100 at RT, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The images were acquired on a Nikon Eclipse TE 2000-U.

Co-immunoprecipitation (Ci)

The physical contact between the GHR and the EGFR, as well as between the uCIS and the EGFR, was studied at the baseline and after induction with GH200, GH1000 or EGF.

Equal amounts of all cell extracts (200 μg of protein) from the fibroblasts cultured in the absence or presence of hGH (200 μg/L) for 15 min were subjected to Ci with the EGFR and CIS antibodies. The immunocomplexes were resolved by SDS/polyacrylamide gel electrophoresis (SDS/PAGE), electrotransferred onto nitrocellulose membranes and the blot was then probed with anti-GHR or anti-EGFR antibodies (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

Results

Our results confirmed our previous findings that in the control fibroblasts, after silencing of CIS mRNA, the protein expression of pSTAT3 was decreased at the baseline and after induction with GH200, and its localization was mainly cytoplasmic. We also confirmed that the GHTD fibroblasts, after silencing of the CIS mRNA, exhibited a remarkable elevation of the protein expression of pSTAT3 at the baseline and after induction with GH200, and the cytoplasmic localization became more intense [20].

The new findings of our study were as follows: in C, after simultaneous silencing of the CIS mRNA and induction with GH200, EGFR changed its localization from exclusively cytoplasmic to localization both in the cytoplasm and on the plasma membrane. In P, the EGFR was localized at first mainly on the plasma membrane after GH200, but after silencing of the CIS mRNA and the administration of GH200, the plasma membrane localization was increased (Figure 1A).

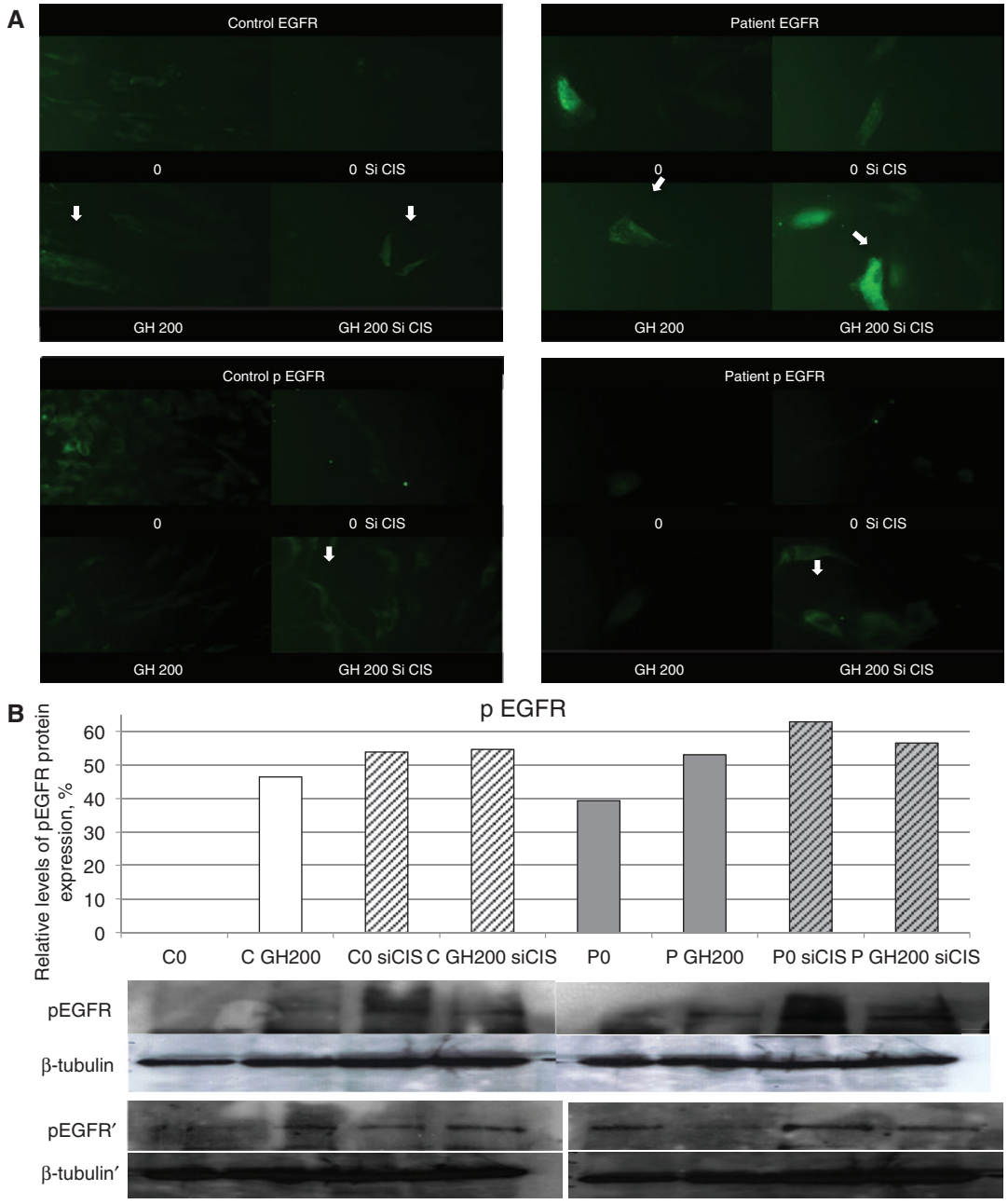

Figure 1: Expression of EGFR and pEGFR at the baseline and after induction with 200 ng/mL hGH, with or without siRNA CIS.
(A) Localization of EGFR and pEGFR as shown with immunofluorescence (If). (B) Western immunoblotting (Wi) of pEGFR. The densitometry measurements are depicted in the histograms.

Also, the protein expression of the pEGFR increased in C and P after silencing of the CIS mRNA, at the baseline and after GH200 (Figure 1B). After simultaneous silencing of the CIS mRNA and induction with GH200, the plasma membrane localization of the pEGFR increased to a greater degree in P as compared to C (Figure 1A).

GH inductions

Ubiquitinated CIS

In the C fibroblasts, as we have previously described [14], after induction with GH1000, the protein expression of uCIS increased, as well as the cytoplasmic localization of

CIS compared to the basal state and after induction with GH200 (Figure 2A and B).

In the P fibroblasts though, after induction with GH1000, the protein expression of uCIS was reduced to similar levels as after induction with GH200 in C, eliminating the overexpression of uCIS observed at the basal state and after induction with GH200. After induction with GH1000, the localization of CIS was mainly in the nucleus, whereas the cytoplasmic localization was reduced compared to that after induction with GH200 or without induction (Figure 2A and B).

Ubiquitin

Ub was overexpressed in the P fibroblasts compared to the C fibroblasts, both at the basal state and after the GH200 and GH1000 inductions. In the C fibroblasts, Ub showed a slight increase after GH1000 when compared to the basal state and after GH200, whereas in the P fibroblasts, Ub was overexpressed from the basal state and after GH200. In the C fibroblasts, Ub showed mainly nuclear expression, whereas in the P fibroblasts, it had mainly nuclear and cytoplasmic expressions (Figure 2A).

STAT3

In C, at the baseline and after GH200 and GH1000, STAT3 showed mainly nuclear localization. In P, STAT3 was localized both in the cytoplasm and in the nucleus at the basal state and after GH200; however, after GH1000, it was also localized on the plasma membrane. In all the states (basal state and after induction with GH200 and GH1000), STAT3 showed increased expression in the P fibroblasts compared to C (Figure 2C).

pSTAT3

In C, the protein expression of pSTAT3 was increased after induction with GH200, whereas after induction with GH1000, it was decreased and was similar to the phosphorylation at the basal state (Figure 2D). The localization of pSTAT3 was observed in the cytoplasm and in the nucleus at the basal state and after induction with GH1000, whereas it was localized on the plasma membrane after induction with GH200 (Figure 2C). In contrast, in P, the protein expression of pSTAT3 and the plasma membrane localization were increased only after induction with GH1000, whereas after induction with GH200, there was almost no phosphorylation of STAT3 (Figure 2C and D).

GHR and EGFR

In C, the protein expression of the GHR and the EGFR was increased after induction with GH200 and decreased after induction with GH1000. In contrast, in P, the protein expression of the GHR and the EGFR was decreased after induction with GH200 and increased after induction with GH1000. After induction with GH200 in C and GH1000 in P, when the protein expression of the GHR and the EGFR was increased, the plasma membrane localization of the two receptors was profound (Figure 2E and F).

pEGFR

After GH200 in C and GH1000 in P, pEGFR also showed increased protein expression and the cytoplasmic and membrane localization of pEGFR were increased (Figure 2G).

EGF induction

CIS, uCIS and ubiquitin

After induction with EGF, in the C fibroblasts, the expression of the total CIS was elevated, with mainly nuclear localization but also with increased cytoplasmic localization as compared to that seen after induction with GH200 or GH1000. The protein expression of uCIS was also increased. In the P fibroblasts, after induction with EGF, the expression of the total CIS was similar to that seen after induction with GH1000 and it had mainly nuclear localization. The protein expression of uCIS was decreased compared to the basal state and after GH200 induction and was similar to that seen after GH1000 (Figure 2A and B).

Ubiquitin

In the C fibroblasts, Ub showed increased expression after induction with EGF compared to the basal state and after the hGH inductions. In the P fibroblasts, Ub showed decreased nuclear and cytoplasmic expressions

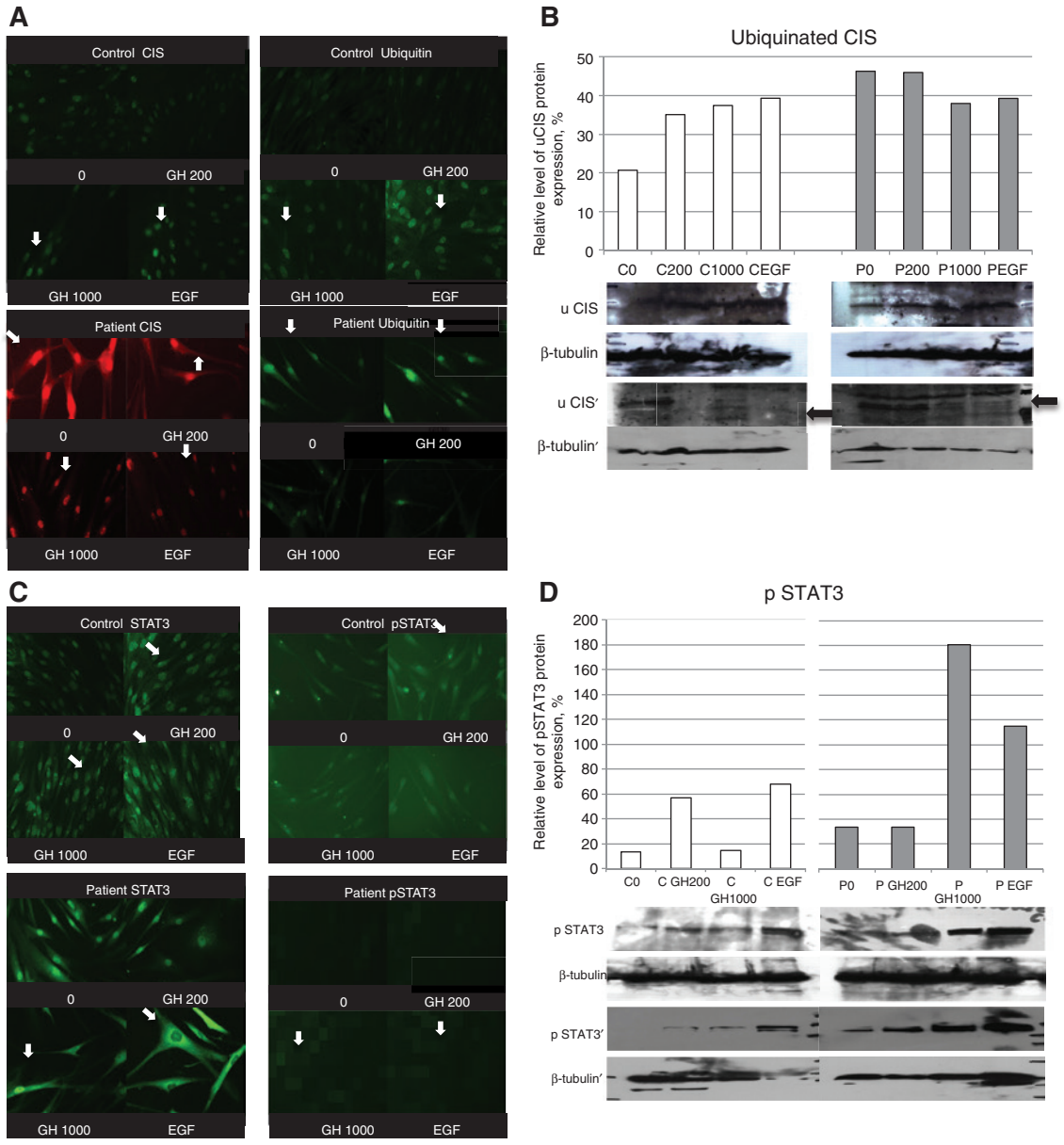

Figure 2: Protein expression (Wi) and localization (If) of proteins of the GH signaling pathway at baseline and after inductions with 200 μg/L GH, 1000 μg/L GH and 50 nM EGF.
(A) If of CIS and ubiquitin, (B) Wi of uCIS, (C) If of STAT3 and pSTAT3, (D) Wi of pSTAT3, (E) Wi and If of GHR, (F) Wi and If of EGFR, (G) Wi and If of pEGFR. The densitometry measurements are depicted in the histograms.

in comparison with the basal state and after GH200 induction (Figure 2A).

STAT3

In the C fibroblasts, after induction with EGF, the expression of STAT3 was increased compared to the basal state and it was mainly localized in the nucleus (Figure 2C). In P, after induction with EGF, the cytoplasmic and membrane expressions of STAT3 were profoundly increased (Figure 2C).

pSTAT3

In C, the protein expression of pSTAT3 was increased after induction with EGF as compared to the basal state, with nuclear and cytoplasmic localizations. In P, the cytoplasmic localization of pSTAT3 was increased after induction with EGF (Figure 2C and D).

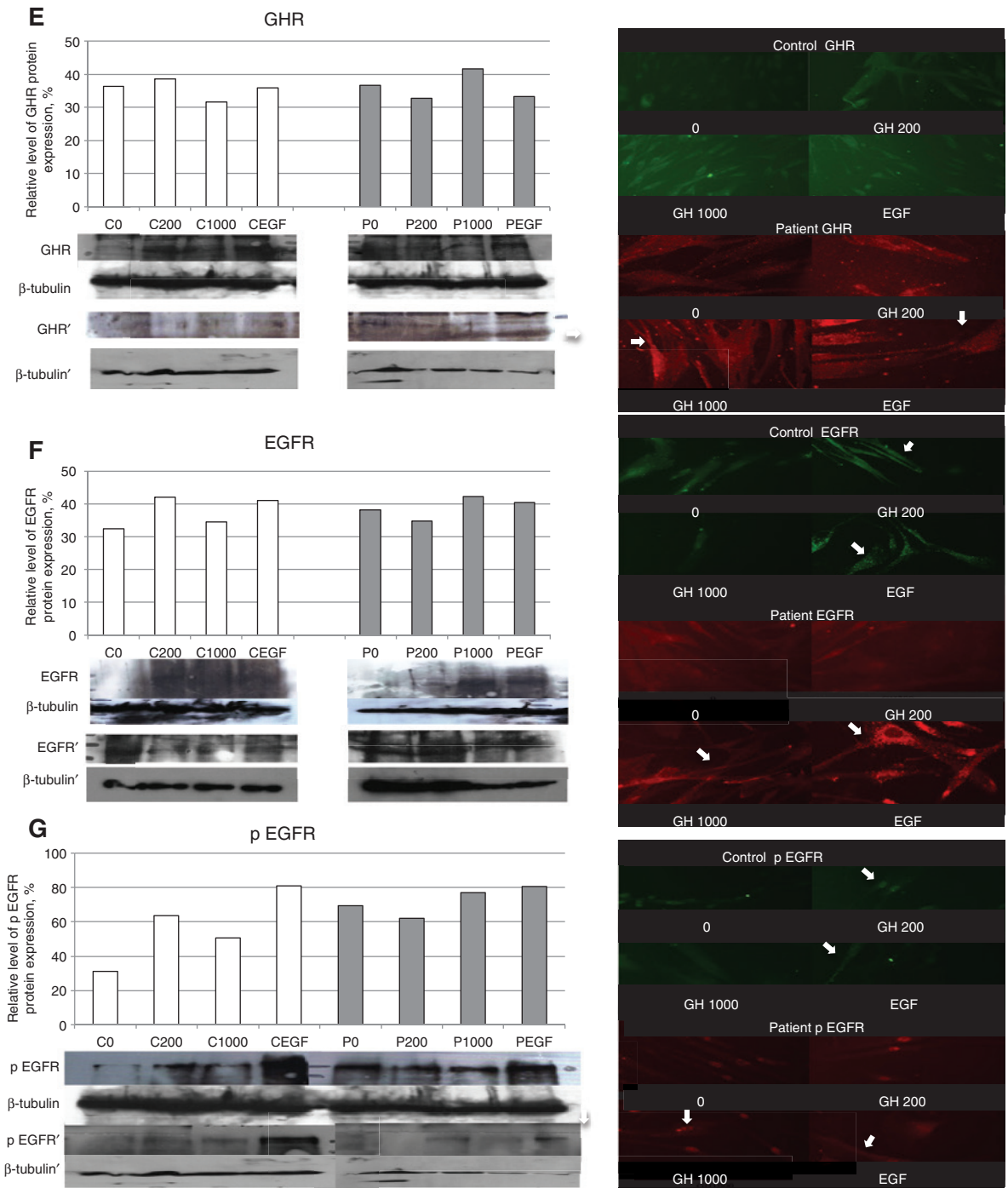

Figure 2 (continued)

GHR and EGFR

In C, the GHR showed no change in its protein expression or in its cytoplasmic localization after induction with EGF compared to the basal state. In P, the GHR, in addition to its cytoplasmic localization, was also present on the plasma membrane after induction with EGF (Figure 2E).

In C, the EGFR was increased similarly after the EGF and GH200 inductions and there was also localization on the plasma membrane in both cases. In P, the protein expression and the membrane localization of EGFR were increased similarly to those seen after GH1000. The membrane localization of EGFR was more profound in P than in C (Figure 2F).

pEGFR

In C, the protein expression of pEGFR was increased even more than after GH200 induction and it was mainly localized on the plasma membrane. In P, the protein expression and the membrane localization of pEGFR were increased. The membrane localization of pEGFR was greater in P than in C (Figure 2G).

Co-immunoprecipitation and double immunofluorescence (Di)

The GHR and EGFR were found to be in physical contact (Ci) after the GH200 and GH1000 inductions in the C fibroblasts, but only after GH1000 in the P fibroblasts. In C, the membrane co-localization (Di) of the receptors was observed after GH200 induction, whereas in contrast, in P, it was observed after GH1000 and EGF inductions (Figure 3A). After induction with GH200 in C and GH1000 in P, the physical contact (Ci) between the proteins EGFR and uCIS decreased as well as the cytoplasmic/membrane co-localization (Di) of the EGFR and CIS (Figure 3B).

Discussion

The results of our previous studies support the hypothesis that impaired GH signaling in children with GHTD may involve the overexpression of uCIS, which causes rapid and excessive translocation of the GHR to the proteasomes for degradation. In the current study, we showed, for the first time, that induction of the GHTD fibroblasts with 1000 μg/L hGH (5 times the amount of hGH needed in control fibroblasts) restored the GHR to the plasma membrane, resulting in physiological GH signaling. This finding provides further evidence that the pathophysiology of GHTD may involve the reversal of the impaired intracellular GH signaling with "pharmacologic"doses of hGH.

Another new finding of this study is the possible involvement of the EGFR in the successful signaling of the GH pathway in the GHTD fibroblasts after induction with hGH. It is well known that GH stimulates tyrosyl phosphorylation of the EGFR, which then activates JAK-STAT signaling [13]. In our current study, as expected, after induction of the control fibroblasts with 200 μg/L hGH, the protein expression and membrane co-localization of the GHR and EGFR, as well as the protein expression of the activated EGFR (pEGFR), increased, facilitating JAK-STAT signaling. It is of interest though that in the GHTD fibroblasts, this co-localization occurred only after induction with hGH 1000 μg/L, inferring that the involvement of the EGFR in the successful JAK-STAT signaling in GHTD may require pharmacologic doses of hGH. It is also noteworthy that after the induction of the fibroblasts with 200 μg/L hGH in C and 1000 μg/L in P, the uCIS and the EGFR showed very little physical contact and co-localization on the membrane and in the cytoplasm, possibly explaining why the EGF receptor is still available on the membrane under the conditions where GH signaling is successful.

It is well known that EGF-induced activation of the EGFR results in STAT3 activation [21, 22]. In accordance with this, in the fibroblasts of C and P, after cellular induction with EGF, the membrane expression of STAT3 was increased, as expected, so that it could be phosphorylated. A new finding, though, was that the membrane expression of STAT3 was more intense in the GHTD fibroblasts after EGF induction. Furthermore, it seems that the EGFR may have a more robust involvement in GH signaling in the GHTD fibroblasts compared to the control fibroblasts. The EGF/EGFR signaling pathway in P in our study seemed to function normally, in contrast to the GH/GHR pathway. This can be inferred from the finding that after EGF induction of the GHTD fibroblasts with the same dose of EGF as in the C fibroblasts, the protein and the membrane expression of the EGF receptor showed no differences between the fibroblasts of C and P. Of particular interest, though, is that the protein and the membrane expression of the phosphorylated EGFR after induction of the C fibroblasts with 200 μg/L hGH were similar to those which occurred only after induction of the GHTD fibroblasts with the higher hGH dose (1000 μg/L hGH). Furthermore, it seems that in the GHTD fibroblasts, the activation of the EGFR with the dose of hGH (1000 μg/L) that induced normal transduction of the GH signal was similar to that which occurred when EGF was the stimulus.

Also, after induction with EGF, the GHR showed increased membrane expression compared to the basal state, which, again, was more profound in the GHTD fibroblasts. It may be possible that in the fibroblasts of P, the crosstalk between the GH and EGF signaling pathways after induction with EGF may be more intense compared to that in the control fibroblasts. In addition, after induction with EGF, the physical contact between the uCIS and the EGFR as well as the co-localization of the CIS and the EGFR on the membrane and in the cytoplasm were noted, especially in the GHTD fibroblasts. This may suggest that uCIS plays a role in the inhibition of the EGF/EGFR pathway. More specifically, the uCIS may either inhibit EGF signaling by binding directly to the EGFR or by binding to the GHR, which we have shown to be in physical contact with

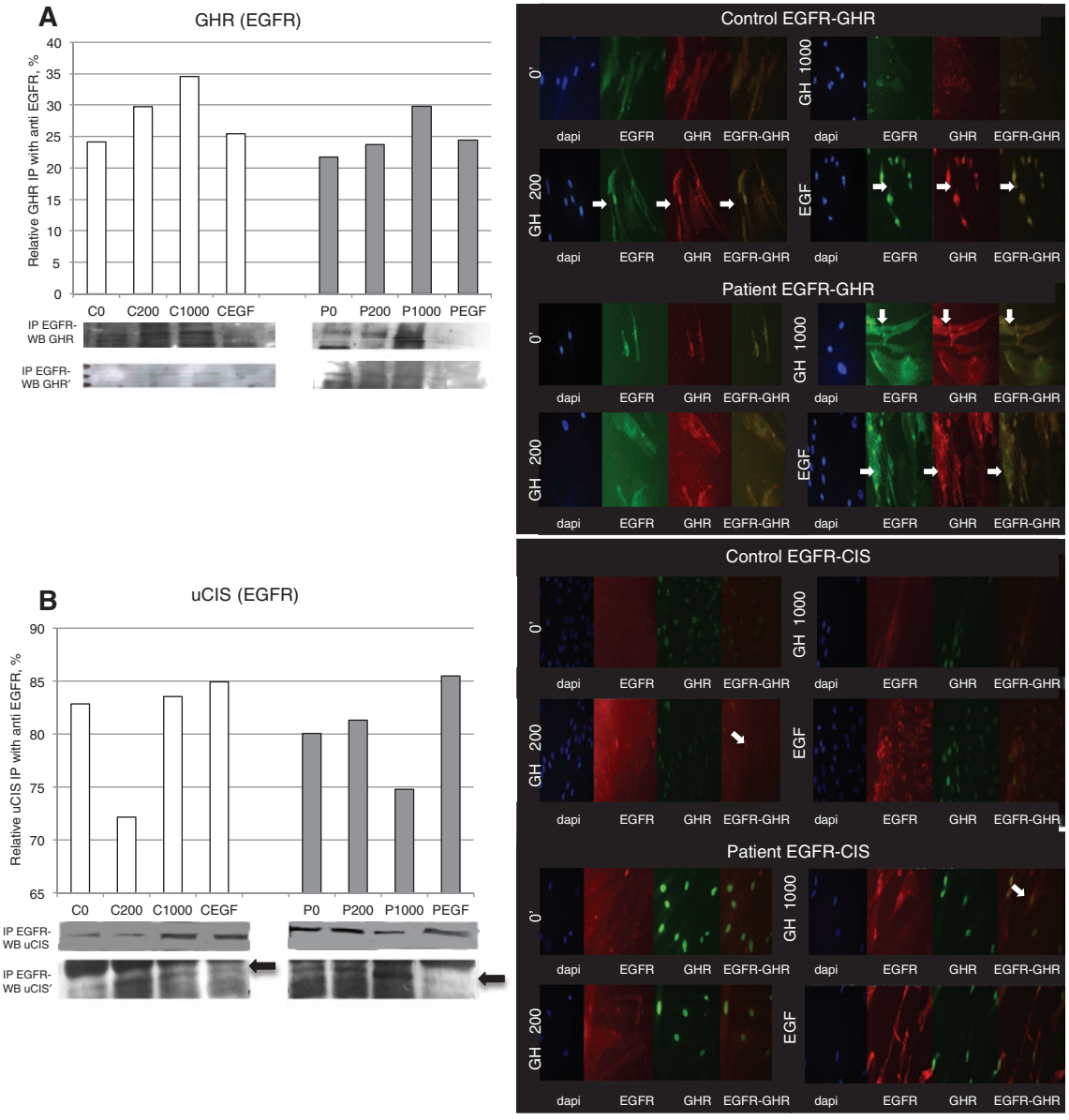

Figure 3: Results from Western immunoblotting (Wi) after co-immunoprecipitation, and double immunofluorescence (dIf), at baseline and after inductions with 200 μg/L hGH, 1000 μg/L hGH or 50 nM EGF.
(A) The precipitation was made with EGFR, and GHR was detected with Wi and co-localization of EGFR-GHR on dIf. (B) The precipitation was made with EGFR, and uCIS was detected with Wi and co-localization of CIS-EGFR on dIf.

the EGFR. After induction with EGF, the control and GHTD fibroblasts exhibited similar levels of uCIS to those seen after the hGH inductions at which GH signaling was successful (200 μg/L hGH in the fibroblasts of the control and 1000 μg/L hGH in the GHTD fibroblasts).

It is well known that CIS is a negative regulator of the GHR and GH signaling [23], and the involvement of SOCS-4 and SOCS-5 in the negative regulation of the EGFR has been documented [24]. In addition, EGF has been shown to induce the expression of CIS in the liver and mammary epithelial cells of mice [25], but our study suggests, for the first time to the best of our knowledge, that uCIS is also a possible negative regulator of the EGFR.

Our study furthers the understanding of GH and EGF signaling, the negative regulation of the GH and EGF pathways and the crosstalk between these two pathways, under normal conditions and in a pathological condition, i.e. GHTD. Successful crosstalk between these two pathways seems to require the membrane localization of both the receptors, GHR and EGFR.

Our findings support the hypothesis that in the fibroblasts of P, the cellular processes that are initiated after

their induction with the higher dose of hGH (1000 μg/L) seem to involve the EGF pathway and may be similar to those induced when exogenous hGH is administered in pharmacologic doses to the P fibroblasts.

In conclusion, our study offers a possible explanation for the successful hGH treatment of Ps, which may occur through the involvement of the EGFR, resulting in increased longitudinal growth (catch-up growth) and a normal final adult height of the children with GHTD.

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References

1. Isaksson OG, Jansson JO, Gause IA. Growth hormone stimulates longitudinal bone growth directly. Science 1982;216:1237–9.
2. Isgaard J, Nilsson A, Lindahl A, Jansson JO, Isaksson OG. Effects of local administration of GH and IGF-1 on longitudinal bone growth in rats. Am J Physiol 1986;250:E367–72.
3. Davidson MB. Effect of growth hormone on carbohydrate and lipid metabolism. Endocr Rev 1987;8:115–31.
4. Kim SO, Loesch K, Wang X, Jiang J, Mei L, et al. A Role for Grb2-associated binder-1 in growth hormone signaling. Endocrinology 2002;143:4856–67.
5. Smit LS, Meyer DJ, Billestrup N, Norstedt G, Schwartz J, et al. The role of the growth hormone (GH) receptor and JAK1 and JAK2 kinases in the activation of Stats 1, 3, and 5 by GH. Mol Endocrinol 1996;10:519–33.
6. Kim SO, Jiang J, Yi W, Fengi GS, Frank SJ. Involvement of the Src homology 2-containing tyrosine phosphatase SHP-2 in growth hormone signalling. J Biol Chem 1998;4:2344–54.
7. Yoshimura A. The CIS family. Negative regulators of JAK-STAT signaling. Cytokine Growth Factor Rev 1998;9:197–204.
8. Leung DW, Spencer SA, Cachlanes G, Hammonds RG, Collins C, et al. Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 1987;330:537–43.
9. Strous GJ, van Kerkhof P, Govers R, Ciechanover A, Schwartz AL. The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. EMBO J 1996;15:3806–12.
10. Landsman T, Waxman DJ. Role of the cytokine-induced SH2-containing protein CIS in growth hormone receptor internalization. J Biol Chem 2005;280:37471–80.
11. Yamauchi T, Ueki K, Tobe K, Tamemoto H, Sekine N, et al. Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. Nature 1997;390:91–6.
12. Kim SO, Houtman JC, Jiang J, Ruppert JM, Bertics PJ, et al. Growth hormone-induced alteration in ErbB-2 phosphorylation status in 3T3–F442A fibroblasts. J Biol Chem 1999;274:36015–24.
13. Huang Y, Kim SO, Jiang J, Frank SJ. Growth hormone-induced phosphorylation of EGF receptor in 3T3–F442A cells: modulation of EGF-induced trafficking and signaling. J Biol Chem 2003;278:18902–13.
14. Kofoed EM, Hwa V, Little B, Woods KA, Buckway CK, et al. Growth hormone insensitivity associated with a STAT5b mutation. N Engl J Med 2003;349:1139–47.
15. Hwa V, Haeusler G, Pratt KL, Little BM, Frisch H, et al. Total absence of functional acid labile subunit, resulting in severe insulin-like growth factor deficiency and moderate growth failure. J Clin Endocrinol Metab 2006;91:1826–31.
16. Woods KA, Camacho-Hübner C, Savage MO, Clark AJ. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N Engl J Med 1996;335:1363–7.
17. Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, et al. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. N Engl J Med 2003;349:2211–22.
18. Ranke MB, Savage MO, Chatelain PG, Preece MA, Rosenfeld RG, et al. Long-term treatment of growth hormone insensitivity syndrome with IGF-I. Results of the European Multicentre Study. The Working Group on Growth Hormone Insensitivity Syndromes. Horm Res 1999;51:128–34.
19. Rojas-Gil AP, Ziros PG, Diaz L, Kletsas D, Basdra EK, et al. Growth hormone/JAK-STAT axis signal-transduction defect. FEBS J 2006;273:3454–66.
20. Rojas Gil AP, Kostopoulou E, Karageorgou I, Kamzelas K, Spiliotis BE. Increased growth hormone receptor (GHR) degradation due to over-expression of cytokine inducible SH2 domain-containing protein (CIS) as a cause of GH transduction defect (GHTD). J Pediatr Endocr Met 2012;25:897–908.
21. Henson ES, Gibson SB. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. Cell Signal 2006;18:2089–97.
22. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene 2006;366:2–16.
23. Ram PA, Waxman DJ. Role of the Cytokine-inducible SH2 protein CIS in desensitization of STAT5b signaling by continuous growth hormone. J Biol Chem 2000;275:39487–96.
24. Kario E, Marmor MD, Adamsky K, Citri A, Amit I, et al. Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling. JBC 2005;280:7038–48.
25. Tonko-Geymayer S, Goupille O, Tonko M, Soratrol C, Yoshimura A, et al. Regulation and function of the cytokine-inducible SH-2 domain proteins, CIS and SOCS3, in mammary epithelial cells. Mol Endocrinol 2002;16:1680–95.