IGF2 Actions on Trophoblast in Human Placenta Are Regulated by the Insulin-Like Growth Factor 2 Receptor, Which Can Function as Both a Signaling and Clearance Receptor

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ABSTRACT

Insulin-like growth factor 2 (IGF2) enhances proliferation and survival of human first-trimester cytotrophoblasts (CTB) by signaling through the insulin-like growth factor 1 receptor (IGF1R). However, the role of the IGF2 receptor (IGF2R) in regulating trophoblast kinetics is unclear: It could act as a clearance receptor for trafficking excess ligand to lysosomes for degradation and/or directly mediate IGF2 signaling. We used an IGF2R knockdown strategy in BeWo cells and placental villous explants to investigate trophoblast proliferation and survival in response to stimulation by IGF. Both IGF1 and IGF2 significantly increased mitosis and reduced apoptosis in serum-starved BeWo cells. Small interfering RNA (siRNA)-mediated knockdown of IGF2R further enhanced IGF2-stimulated mitosis (P < 0.001), and IGF2-mediated rescue of apoptosis (P < 0.001) in these cells. Leu27IGF2, an IGF2 analogue that binds to IGF2R but not IGF1R, also protected IGF2R-expressing BeWo cells from apoptosis but did not increase mitosis. IGF treatment of term placental villous explants with reduced syncytial expression of IGF2R increased CTB proliferation (P < 0.001) and decreased apoptosis (P < 0.001) compared to untreated controls. Moreover, IGF2-mediated rescue of CTB apoptosis was significantly greater than that in tissue with normal IGF2R expression. Leu27IGF2 promoted mitogenesis and survival only in explants with intact IGF2R expression. Given that altered CTB turnover is observed in pregnancies complicated by fetal growth restriction, the development of strategies to manipulate the IGF2R signaling axis in the syncytiotrophoblast may provide a therapeutic avenue for treating this condition.

apoptosis, IGF, insulin-like growth factor receptor, placenta, pregnancy, proliferation, trophoblast

INTRODUCTION

The insulin-like growth factors 1 and 2 (IGF1 and IGF2, respectively) are important regulators of fetal and placental development in both mice and humans. Igf1-null mice weigh 40% less than wild-type pups [1, 2], and ablation of Igf2 leads to concurrent fetal and placental growth restriction [3–5]. Moreover, the generation of mice lacking the labyrinthine trophoblast-specific Igf2P0 transcript has confirmed a role for IGF2 in placental development. In these mice, placental growth restriction was recorded at Embryonic Day (E) 12, and fetal growth restriction (FGR) developed at E16. Proportional reductions in the size of placental compartments lead to altered exchange barrier morphology and a reduction in passive permeability.

In humans, IGF1 concentration in cord blood at term is positively correlated with birth weight [6, 7], and a partial deletion of the IGF1 gene also results in severe FGR [8]. In uncomplicated pregnancies, placental IGF production is evident from approximately 6 wk of gestation [9, 10]; thus, growth and development beyond this time are regulated by both maternally and fetally derived IGFS.

Using explants of first-trimester human placenta, we have shown that maternal IGF1 and IGF2 can signal across the syncytiotrophoblast—tissue layer of trophoblast—to stimulate proliferation and survival of underlying cytotrophoblast (CTB) [11]. This is an important determinant in the expansion of the placental syncytium and the generation of an exchange barrier with sufficient surface area to meet the nutritional and oxygen needs of the growing fetus. Several in vitro studies suggest that IGFS may also regulate placental development by promoting trophoblast migration and invasion [12, 13] into decidua and myometrium, thus contributing to remodeling of the uterine spiral arteries and ensuring optimal delivery of maternal blood to the placenta.

In the human placenta, the IGF1 receptor (IGF1R) is expressed by the syncytiotrophoblast and underlying CTB [14, 15], and expression of the IGF2 receptor (IGF2R; cation-dependent mannose-6-phosphate receptor) has been described in the syncytiotrophoblast at term [16, 17] and in a first-trimester trophoblast cell line [18]. The actions of both IGFS are chiefly mediated via the IGF1R, ligation of which induces receptor autophosphorylation, phosphorylation of insulin receptor substrate 1 (IRS1) and activation of the phosphoinositide 3-kinase and mitogen-activated protein kinase (MAPK) pathways. However, IGF2 can also bind to the IGF2R, which acts primarily to target excess IGF2 for degradation in the lysosomes. Consequently, Igf2-null mice are characterized by elevated circulating levels of IGF2, enlarged placentas, and somatic cell overgrowth [19]. Nevertheless, accumulating evidence suggests that IGF2R also has a role in IGF2 signaling [20–23]. Indeed, IGF2 can stimulate cell migration via this receptor [18], though to our knowledge its role in the regulation of trophoblast kinetics has not been reported.

Failure of the placenta to develop optimally can lead to miscarriage [24], FGR [25], preeclampsia [26], and preterm birth [27]. Because small size at birth is associated with an
increased risk of elevated serum cholesterol, cardiovascular disease, stroke, type 2 diabetes, adiposity, and insulin resistance in later life [28–30], development of therapeutic interventions to promote placental and fetal growth in utero could enhance long-term health and well-being. Systemic administration of IGF1 or IGF2 in pregnancy is ill-advised, because IGF1R is ubiquitously expressed, implying a potential for multiple off-target effects. However, one solution would be to enhance the local concentration of IGF at the maternofetal interface, which might be achieved by reducing IGF2R-mediated clearance of IGF2. For the present study, we used an IGF2 analogue, LeuIGF2, that binds only to IGF2R to investigate the role of IGF2R in regulating human trophoblast kinetics and thus determine the consequence of decreasing IGF2R expression.

MATERIALS AND METHODS

Cell Culture

BeWo cells (a human choriocarcinoma cell line) were cultured in a 1:1 ratio of Dulbecco modified Eagle medium (DMEM) and Ham F12 (Lonza) containing glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% (v/v) fetal bovine serum (Fisher Scientific). Cells were maintained in 95% air and 5% CO₂ at 37°C.

Tissue Culture

Term placentas (37–42 wk of gestation) were obtained from uncomplicated pregnancies within 30 min of vaginal or cesarean delivery. Written informed consent was obtained, and the study was performed in accordance with local ethics committee approval. Villous tissue was randomly sampled, and biopsy specimens (2 cm³) were washed several times in serum-free culture medium and dissected into 5-mm³ explants under sterile conditions. Explants (n = 1 per well) were submerged in 1 ml of a 1:1 ratio of DMEM and Ham F12 medium containing glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% (v/v) fetal bovine serum in 24-well culture plates precoated with agarose (1% w/v). Explants were maintained in 95% air and 5% CO₂ at 37°C for up to 72 h.

Transfections

Small interfering RNA (siRNA)-mediated knockdown of IGF2R was performed by targeting a site located 2050 nucleotides downstream of the start codon of IGF2R, using siRNA sequences previously published, double-stranded, sequence-specific siRNA (21) (5’-GGGUUUUUCUUUGACUUAUtt-3’ and 5’-AUAAGUCUAAA GAAAACCtG-3’). Two nontargeting (NT) double-stranded sequences (NT1, 5’-ACGGUGACACCGGUCCGAAAdTdT-3’; NT2, 5’-UUCUCCG AACGGUGACACCGUttTdT-3’) were used as controls (Ambion).

BeWo cells. Cells were transfected by electroporation using an Amaxa Nucleofector machine (program X-005) and Amixa cell line Nucleofector kit L (Lonza) as previously described [31]. Additional controls included cells that were not subjected to electroporation and mock-transfected cells that were subjected to electroporation in the absence of siRNA.

Placental explants. siRNA sequences added to the culture medium of term placental explants in the absence of transfection reagents or electroporation spontaneously accumulate in the syncytiotrophoblast layer as confirmed by preclinical experiments using fluorescent siRNA sequences (Sigmofluor fluorescent siRNA; 400 nM; Dharmacon); thus, knockdown of IGF2R in tissue was achieved by adding the specific IGF2R siRNA sequence (100 nM). The scrambled sequence (NT1, 100 nM) was used as a control. The efficiency of knockdown was determined by quantitative real-time PCR (qPCR), immunohistochemistry, and Western blot analysis.

Analysis of IGF2R mRNA and Protein Expression

Quantitative real-time PCR. Total RNA from BeWo cells was extracted, quantified, and reverse transcribed (100 ng from each sample) as previously described [32]. IGF2R mRNA was quantified by qPCR using previously published primer sequences (forward primer, 5’-GGGAGAGGGCATACAGCGG-3’, reverse primer, 5’-TGGTGGCGAGCATGTC-3’, Invitrogen) [21] and Stratagene Brilliant SYBR Green I qPCR Master Mix, with 5-carboxy-X-rhodamine as a passive reference dye in a Stratagene MX3000P real-time PCR machine. IGF2R mRNA expression in triplicate samples was quantified against a standard curve constructed from the cDNA of human reference RNA (Stratagene). Expression was normalized to human reference cDNA samples generated in the same RT reaction, which served as an internal control, as previously described [33]. The identity of the amplified product was determined by DNA sequencing and was confirmed to be IGF2R (data not shown).

Immunoprecipitation and Western blot analysis. Culture dishes containing BeWo cells were placed on ice and washed twice with PBS. Following the addition of lysis buffer containing protease and phosphatase inhibitors (1X PBS, 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 1 mM Na3VO4, 1 mM PMSF, and 10 µg/ml of aprotinin), cells were scraped from their dishes. The resulting lysates were incubated on ice for a further 20 min and then centrifuged (5000 × g, 10 min). Aliquots of the supernatants (150 µg of protein) were preclariﬁed by incubation with protein G-coated Sepharose beads (20 µl, 30 min. 4°C, Sigma-Aldrich) that had been prewashed in ice-cold PBS. Samples were centrifuged (5000 × g, 30 sec) to pellet the beads, and supernatants were transferred to new tubes containing fresh, prewashed beads (20 µg) and a mouse polyclonal anti-IGF2 receptor antibody (2 µg; Abnova). Samples were incubated on a rotor for 4 h at 4°C and centrifuged again (5000 × g, 30 sec) to pellet the beads. Pellets were washed twice in ice-cold PBS, resuspended in SDS loading buffer (50 µl), and boiled for 5 min. Samples were then centrifuged again (5000 × g, 1 min), and equal amounts of protein (20 µg) were separated by SDS-PAGE on 6% gels under nonreducing conditions and transferred to nitrocellulose membranes. These FIG. 1. IGF2R knockdown in BeWo cells. BeWo cells were electroporated in the absence or presence of two different NT siRNA sequences (NT1 and NT2; 100 nM) or an siRNA sequence specific for IGF2R (IGF2R; 50 or 100 nM).Following 48 h in culture, IGF2R mRNA expression was quantified by qPCR (A; mean + SEM, n = 3), and protein expression was analyzed by Western blot analysis (B) and immunocytochemistry (C). Images are representative of three independent experiments. Bar = 25 µm. PI, propidium iodide.
membranes were blocked (Tris-buffered saline [TBS], 0.1% v/v Tween 20 and 5% w/v milk powder) for 1 h, then probed with a polyclonal goat anti-human IGF2R antibody (overnight, 1:100; R&D Systems) or a monoclonal mouse anti-human β-actin (overnight, 1:1000; Sigma-Aldrich) prepared in TBS-Tween 20 (0.5% w/v milk), followed by an anti-goat or an anti-mouse horseradish peroxidase-conjugated secondary antibody (2 h, 1:1250; DakoCytomation). Following washing, proteins were detected by enhanced chemiluminescence (GE Healthcare).

Effect of IGF Treatments on Trophoblast Cell Turnover

BeWo cells. Following transfection with siRNA, BeWo cells were plated onto glass cover slips or into 96-well plates and cultured in serum-containing medium for 24 h, then serum-starved for 24 h. Recombinant human IGF1 (Sigma-Aldrich), IGF2 (Sigma-Aldrich), or Leu27IGF2 (10 nM; Gropep) was added to the cells, which were maintained under serum-free conditions for 24 or 48 h and assayed for proliferation (by counting mitotic nuclei) or apoptosis (TUNEL and caspase-3/7 activity) and necrosis (lactate dehydrogenase [LDH] assay).

Placental explants. Term placental explants were cultured in serum-containing medium for 24 h in the absence or presence of siRNA, then transferred to serum-free medium for a further 24 h. IGF1, IGF2, or Leu27IGF2 (10 nM; Gropep) was added, and 24 h later, tissue was fixed in paraformaldehyde (4% w/v in PBS overnight) and assayed for proliferation and apoptosis by immunohistochemical analysis of MKI67 and M30 expression, respectively.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

BeWo cells were fixed with 4% (w/v) paraformaldehyde in PBS for 20 min, permeabilized with 0.1% (v/v) Triton-X in 0.1% (w/v) sodium citrate in H2O for 8 min, then washed in PBS. Cover slips were incubated (90 min, 37°C) with working TUNEL reagent solution prepared according to the manufacturer’s instructions (In Situ Cell Death Detection Kit [TUNEL]; Roche). The enzyme provided was diluted 1:5 with PBS to reduce background fluorescence. Cover slips were washed three times in PBS, mounted using Vectashield mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories), and stored at 4°C in the dark. Quantification of the number of TUNEL-positive cells was performed in a blinded fashion using a Zeiss fluorescence microscope. In each independent experiment, 10 random images were captured, counted, and used to determine a mean number of TUNEL-positive cells.

Active Caspase-3/7 Assay

Triplicate wells of BeWo cells were incubated with a proluminescent Z-DEVD-luciferin substrate, specific for caspase-3/7, for 1 h at room temperature according to the manufacturer’s instructions (Caspase-Glo kit; Promega Corp.). In the presence of active caspase-3/7, the substrate is cleaved to generate light. Thus, relative caspase activity in each well was determined using a lumimeter.

Lactate Dehydrogenase Assay

The level of necrosis was assessed using a commercially available kit (Roche) to measure LDH release into the culture medium. Media were centrifuged (1000 × g, 5 min) to remove cell debris, and then triplicate 100-μl aliquots from each sample were combined with 100 μl of the reaction solution provided in a 96-well plate. The plate was incubated for 30 min at room temperature in the dark, and after the addition of HCl (1 M, 5 μl) to stop the reaction, absorbance at 492 nm was measured using a plate reader.

FIG. 2. The effect of IGF1, IGF2, and Leu27IGF2 on the mitosis and survival of BeWo cells with and without reduced IGF2R expression. BeWo cells were transfected with NT or IGF2R-specific siRNA sequences and cultured for 24 h. Cells were then serum-starved for a further 24 h and treated with vehicle (control), IGF1 (10 nM), IGF2 (10 nM), or Leu27IGF2 (10 nM) for 24 or 48 h. Proliferation was assessed after 24 h by determining the percentage of mitotic cells (A; n = 8 independent experiments). Apoptosis and necrosis were assessed after 48 h by measuring TUNEL (B; n = 7 independent experiments), caspase-3/7 activity (C; n = 6 independent experiments), or LDH release into the culture medium (D; n = 6 independent experiments). Data are shown as the mean ± SEM. A lowercase letter a indicates a significant difference (P < 0.05) from vehicle-treated cells by one-way ANOVA with least significant difference post hoc testing. A lowercase letter b indicates a significant difference (P < 0.05) from IGF2-treated cells transfected with a NT siRNA sequence by t-test.
**Immunocytochemistry**

BeWo cells were fixed with 4% (w/v) paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton-X in PBS for 8 min. After washing in PBS, non-specific binding sites were blocked by incubating cells with 5% (w/v) bovine serum albumin (BSA) in PBS for 30 min. Cells were then incubated with a polyclonal goat anti-human IGF2R antibody (1:60; R&D Systems) for 1 h at room temperature. Cells were washed three times in PBS and incubated for 1 h with a rabbit anti-goat immunoglobulin (IgG) G antibody (1:200; DakoCytomation). After a further three PBS washes, cells were incubated with a streptavidin-fluorescein isothiocyanate conjugate (1:50; DakoCytomation) for 45 min in the dark. Cover slips were mounted using Vectashield mounting medium containing propidium iodide or DAPI and stored at 4°C in the dark until viewed. Slides were analyzed using either a Bio-Rad Radiance 2100 confocal microscope with a 10× or a 40× oil-immersion objective lens and LaserSharp 2000 image analysis software or a Zeiss fluorescence microscope.

**Immunohistochemistry**

Sections of wax-embedded, term placental explants (thickness, 5 μm) were deparaffinized in Histoclear (Fisher) and alcohol, then microwaved for 10 min in sodium citrate buffer (0.01 M) containing 0.05% (v/v) Tween 20 (pH 6.0) to facilitate antigen unmasking. After cooling, endogenous peroxide activity was blocked by placing the slides in methanol containing 0.4% (v/v) HCl and 0.5% (v/v) hydrogen peroxide for 30 min. Tissue sections were washed three times in 0.05 M TBS and blocked with 5% (w/v) BSA in TBS for 30 min. Primary antibodies, diluted to the required concentration with 0.05 M TBS (IGF2R [R&D Systems], 1:100; MKI67 [DakoCytomation], 1:50; M30 CytoDEATH antibody [Roche], 1:50; control IgG [DakoCytomation], neat), were applied to the tissue sections, which were incubated overnight at 4°C in a humidity chamber. Slides were washed (three washes in TBS), and the secondary antibodies, diluted in TBS (biotinylated rabbit anti-goat IgG, 1:500; biotinylated goat anti-mouse IgG, 1:200; both from DakoCytomation), were applied for 30 min at room temperature. Slides were washed again (three washes in TBS) and incubated with avidin peroxidase (5 Ci/ml in 0.125 M TBS; Sigma-Aldrich) for 30 min at room temperature. Slides were washed in TBS and incubated for 1–5 min with 0.05% (w/v) diaminobenzidine and 0.015% (v/v) hydrogen peroxide (Sigma-Aldrich). Slides were washed in dH₂O, counterstained with hematoxylin, rehydrated in alcohol and Histoclear, and mounted in DPX (Sigma-Aldrich). In each independent experiment, six random images were captured, counted, and used to determine a mean number of MKI67- or M30-positive cells.

**Data Analysis**

Normally distributed data were analyzed by ANOVA with Bonferroni post hoc test or with a Student t-test using GraphPad Prism software (Version 4). Data are expressed as the mean ± SEM from at least three independent experiments. A P value of 0.05 was considered to be significant.

**RESULTS**

Two approaches were used to investigate whether IGF2R is involved in regulating the response of the trophoblast cell line BeWo to IGF: siRNA-mediated knockdown of IGF2R and treatment with Leu²⁷IGF2, an IGF2 analogue selective for IGF2R.

### siRNA-Mediated Knockdown of IGF2R in BeWo Cells

Neither mock transfection nor transfection with the two NT siRNA sequences (100 nM) significantly affected IGF2R mRNA expression. However, the IGF2R-specific siRNA sequences reduced mRNA (50nM, 84% reduction vs. NT; 100 nM, 79% reduction) and protein expression (50 nM, 49% reduction; 100 nM, 46% reduction) after 48 h (Fig. 1). All subsequent experiments used 50 nM IGF2R siRNA, with 100 nM NT1 serving as a control.

### Manipulation of IGF2R Expression Alters IGF2 Function in BeWo Cells

Serum-starved BeWo cells transfected with NT or IGF2R-specific siRNA sequences were treated with IGF1, IGF2, or Leu²⁷IGF2, and proliferation and survival were assessed at 24 and 48 h, respectively. As expected, the number of mitotic cells...
reduced IGF2R expression. Or Leu27IGF2 (10 nM) for 24 h. Proliferation and apoptosis were assessed for 24 h, then treated with vehicle (control), IGF1 (10 nM), IGF2 (10 nM), sequences and cultured for 24 h. Next, explants were then serum-starved survival of villous CTB with and without reduced IGF2R expression. Term placental explants were transfected with NT or IGF2R-specific siRNA 

FIG. 4. The effect of IGF1, IGF2, and Leu27IGF2 on the mitosis and survival of villous CTB with and without reduced IGF2R expression. Term placental explants were transfected with NT or IGF2R-specific siRNA sequences and cultured for 24 h. Next, explants were then serum-starved for 24 h, then treated with vehicle (control), IGF1 (10 nM), IGF2 (10 nM), or Leu27IGF2 (10 nM) for 24 h. Proliferation and apoptosis were assessed by immunohistochemical analysis of MKI67-positive (A) and M30-positive (B) CTB, respectively. Data are presented as the mean + SEM (n = 8 independent experiments). A lowercase letter a indicates a significant difference (P < 0.05) from vehicle-treated explants by one-way ANOVA with least significant difference post hoc testing. A lowercase letter b indicates a significant difference (P < 0.05) from IGF2-treated explants transfected with a siRNA sequence by t-test.

observed after stimulation with either IGF1 or IGF2 was significantly more than the number in untreated controls, irrespective of the level of IGF2R expression (Fig. 2A). However, Leu27IGF2 had no effect on the mitotic count in cells transfected with NT or IGF2R-specific siRNA sequences.

Both IGF1 and IGF2 are established survival factors for trophoblast [11], and indeed, both factors rescued IGF2R-replete and IGF2R-depleted BeWo cells from apoptosis induced by serum starvation (Fig. 2, B and C). Interestingly, Leu27IGF2 reduced the number of TUNEL-positive cells (Fig. 2B) and caspase-3 activity (Fig. 2C) in cells with an intact IGF2R expression profile, but not after IGF2R expression had been knocked down. Similarly, all IGF treatments rescued IGF2R-replete cells from necrosis induced by serum starvation (Fig. 2D); however, Leu27IGF2 did not protect cells with reduced IGF2R expression.

The IGF2 (or Leu27IGF2) activation of IGF2R might signal directly to enhance cell survival, in which case knockdown of IGF2R would eliminate the response. However, IGF2R is more typically regarded as an IGF2 clearance receptor, so reducing its expression could result in an increase of IGF2 bioavailability and more signaling through IGF1R. Indeed, after IGF2R expression was reduced, IGF2 had a significantly greater effect on mitosis (Fig. 2A) and survival (Fig. 2, B and C). However, IGF2-mediated rescue of necrosis was not enhanced (Fig. 2D).

**IGF2R Activation Alters Trophoblast Function in Term Placental Explants and Knockdown Enhances IGF2-Mediated Rescue of Apoptosis**

Receptor function in placental tissue requires independent evaluation, because the system has a postmitotic, IGF2R-positive syncytiotrophoblast layer above a proliferative CTB progenitor layer. Both layers express the two receptors, IGF1R and IGF2R, but because IGF2R is expressed more highly at term than in the first trimester (Fig. 3A), the effect of IGF2R knockdown on the function of trophoblast was assessed in explants of human term plenta. In the absence of delivery reagents or electroporation, siRNA spontaneously enters the syncytiotrophoblast layer of first-trimester placental explants [31], and this effect was verified in term placenta by tracking a fluorescent siRNA into syncytiotrophoblast in explants (data not shown). Incubation of explants with NT siRNA had no effect, whereas the IGF2R-specific siRNA sequence reduced IGF2R mRNA and protein expression. This effect was predominantly in syncytiotrophoblast, because immunohistochemical analysis showed sustained expression in CTB (Fig. 3C). Maximal IGF2R knockdown was observed after 48 h, so this time point was used in subsequent experiments.

Serum-starved explants of villous tissue were treated with IGF1, IGF2, or Leu27IGF2 for 24 h, and then proliferation and apoptosis were assessed by immunostaining for MKI67 and M30, respectively. In explants transfected with NT siRNA, both IGF1 and IGF2 significantly increased MKI67-positive CTB (Fig. 4A) when compared to untreated tissue. Unlike in BeWo cells, Leu27IGF2 promoted a similar increase in proliferation, and this effect was abolished by reducing syncytiotrophoblast IGF2R expression (Fig. 4A). As in BeWo cells, IGF2R knockdown augmented the proliferative stimulus delivered by IGF2, but this effect was weaker in explants and did not reach statistical significance.

Analysis of CTB apoptosis revealed findings similar to those in BeWo cells. In comparison to untreated tissue, IGF1, IGF2, and Leu27IGF2 significantly reduced the number of M30-positive CTB in explants transfected with NT siRNA (Fig. 4B), and the effect of Leu27IGF2 was dependent on the level of IGF2R. Furthermore, in tissue with reduced IGF2R, IGF2-mediated rescue of apoptosis was further enhanced compared to that in tissue with normal IGF2R expression.

**DISCUSSION**

Using the IGF2 analogue Leu27IGF2, the present study has shown that IGF2 can influence trophoblast kinetics through activation of IGF2R (Fig. 5A). Our data also indicate that IGF2R functions as a clearance receptor, because IGF2R knockdown enhanced IGF2 activity, thereby suggesting that IGF2 signaling can be redirected through IGF1R (Fig. 5B).

Reducing the level of IGF2R in BeWo cells enhances IGF2R-mediated rescue from apoptosis. Mitotic counts after IGF2-stimulation are also enhanced, in keeping with data from another trophoblast cell line, JEG3 [34, 35]. Control experi-
mments showed that the effects of IGF1, which does not bind IGF2R, are unchanged after knockdown of this receptor. These findings are consistent with an increase in IGF2 signaling through IGF1R caused by increased IGF2 bioavailability, perhaps as the result of decreased IGF2R-mediated internalization of bound ligand. In mice lacking IGF2R, both embryos and placentas are larger [36, 37], consistent with this mechanism. However, Leu27IGF2 treatment of BeWo cells with an intact IGF2R expression profile caused a significant decrease in apoptosis, suggesting the possibility that activation of this receptor in trophoblast might also directly regulate antiapoptotic signaling pathways.

The effects of IGF2R knockdown on the proliferation of trophoblast in term placental tissue were similar, but not identical, to those of BeWo cells. Here, the protocol used was effective in reducing IGF2R expression in the syncytiut but not in the underlying CTB, and this allowed us to probe receptor function in the context of postmitotic syncytiotrophoblast. We [11] and others [38] have previously suggested that the syncytiut transduces signals from the maternal circulation to the proliferative layers of the placenta. Here, the proportion of CTB in cycle (MKI67-positive cells) after IGF2 treatment increased, and this occurred to a slightly greater, though not statistically significant, extent than that observed in tissue with intact syncytiut IGF2R expression.

The IGF2R-specific ligand Leu27IGF2 stimulated CTB proliferation only in receptor-replete tissue and did not stimulate proliferation at all in BeWo cells; this might suggest trans-syncytial transport of the normal ligand IGF2 occurs via IGF2R internalization in tissue and may be followed by presentation to IGF1R on CTB. Further experiments are planned to explore this hypothesis. In keeping with this suggestion, rescue of CTB apoptosis in response to Leu27IGF2 was lost after the receptor had been depleted from the syncytiut. If IGF2R can play roles in both transcellular trafficking and the bioavailability of IGF2 at the syncytiut surface, then further questions arise as to how a balance is maintained between these dual roles and the complementary functions of IGF1R.

Our results are in keeping with the observation that IGF2 protects first-trimester CTB from apoptosis induced by serum starvation [11]. However, it is not clear if this effect is mediated by IGF2R, because IGF2R expression is relatively low in first-trimester placenta. CTB proliferation is more rapid in the first trimester than at term [35], and increasing IGF2R levels as term approaches are more consistent with a function in enhancing cell survival at later stages of pregnancy rather than in modulating proliferation. Decreased expression of IGF2R in other cell types can reduce apoptosis in response to hypoxia or tumor necrosis factor-α [39].

Our findings complement studies in the guinea pig, in which systemic administration of Leu27IGF2 increased the volume of the placental labyrinth, increased the trophoblast surface area, and decreased barrier thickness for exchange, resulting in increased fetal weights at term and higher fetal plasma amino acid concentrations [40]. Whether the effect of Leu27IGF2 on guinea pig placentation was achieved by signaling through IGF2R or simply via displacement of IGF2 from this receptor is currently unclear; the two mechanisms are not mutually exclusive. In HEK293 cells, ligation of IGF2R by IGF2 stimulates sphingosine kinase activation and S1P production, leading to G protein-dependent activation of MAPK3/1 signaling [20, 21]. Similarly, Leu27IGF2 has been shown to increase migration of HTR-8 trophoblast cells via MAPK3/1 activation [18] and induce apoptosis of cardiomyoblasts via activation of Gqα and phospholipase C-β [41], implicating IGF2R as a functional signaling receptor.

The present study has shown that manipulation of IGF2R expression has the potential to beneficially affect IGF2 function in the placenta, because IGF2-mediated rescue from apoptosis is increased. These findings may be of clinical relevance, because in rats, FGR has been characterized by down-regulation of the IGF2 signaling pathway and increased placental apoptosis [42]. A higher proportion of apoptotic cells are present in placentas from pregnancies complicated by FGR compared to normal pregnancies [43], although the distribution and density of IGF1R remain unchanged [14]. Therefore, targeted interventions that decrease IGF2R function...
in the placenta may provide a novel approach to treating pregnancies complicated by growth restriction.

REFERENCES


