Published in final edited form as:

Apoptosis. 2013 March ; 18(3): 361–368. doi:10.1007/s10495-012-0793-3.

Regulation of retinal endothelial cell apoptosis through activation of the IGFBP-3 receptor

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Abstract

The goal of this study was to investigate whether insulin-like growth factor binding protein-3 receptor (IGFBP-3 receptor) is required for IGFBP-3 to inhibit retinal endothelial cell (REC) apoptosis. REC were grown in normal glucose (5 mM) or high glucose medium (25 mM) for 3 days. Once cells reached confluence, they were transfected with an endothelial-specific IGFBP-3 plasmid DNA (non-IGF binding; IGFBP-3 NB) at 1 µg/ml for 24 h. Cell proteins were extracted and analyzed for IGFBP-3 receptor expression by Western blotting or use in coimmunoprecipitation or co-localization experiments for detection of IGFBP-3 and IGFBP-3 receptor binding. REC were also transfected with or without IGFBP-3 receptor siRNA before IGFBP-3NB plasmid DNA transfection. Cell lysates were processed for a cell death ELISA, a cleaved caspase 3 ELISA, and Western blotting to measure key pro- and anti-apoptotic markers: Bcl-xL, Bax, Cytochrome C and Akt. The IGFBP-3 receptor is present on REC. Overexpression of IGFBP-3 in REC significantly increased protein levels of IGFBP-3 receptor ($p < 0.05$). Significant increases in cell death were found in cells transfected with IGFBP-3 receptor siRNA versus not treated samples ($p < 0.05$). Data suggest that IGFBP-3 inhibits retinal endothelial cell death through activation of an IGFBP-3 receptor in a hyperglycemic environment. This is the first demonstration of the involvement of IGFBP-3 receptor in inhibition of REC cell death. Future studies will investigate the mechanism by which IGFBP-3 receptor may inhibit retinal endothelial cell death.

Keywords

Apoptosis; IGFBP-3; IGFBP-3 receptor; Retinal endothelial cell

Introduction

Insulin-like growth factor binding protein 3 (IGFBP-3) is the predominant IGFBP family member to carry insulin-like growth factor 1 (IGF-1) in the circulation [1]. IGFBP-3 can bind the circulating IGF-1 with high affinity and specificity, modulate IGF-1 action by increasing IGF half-life or inhibiting its function by sequestering the peptide and preventing IGF binding to its receptor [1, 2]. IGFBP-3 has also been recently reported to have direct intrinsic actions by binding to several addition molecules and function independent of IGF-1 [3]. IGFBP-3 can be induced by tumor necrosis factor- α , transforming growth factor beta (TGF- β), vitamin D, retinoids, and anti-estrogens [4–7]. It can modulate cell proliferation by adjusting levels of p53, preventing DNA damage, and modulating NF- κ B activities [8, 9]. These cumulative reports have suggested that IGFBP-3 has important biological significance in controlling cell growth, transformation and survival, independent of its actions on IGF-1 regulation. However, the underlying molecular mechanisms involved in these biological actions remain to be characterized.

IGF-1 and IGFBP-3 have been reported to block both physiologic and pathologic angiogenesis in diseases such as diabetic retinopathy [10–12]. It was reported that abnormally low levels of IGF-1 and IGFBP-3 were found in the serum of diabetic patients compared to controls; IGF-1 and IGFBP-3 levels decreased significantly with age in diabetic patients [13]. Some have suggested that a rescue of IGFBP-3 may prevent the progression of diabetic retinopathy [14]. Previously, we found that IGFBP-3 decreases apoptosis in retinal endothelial cells in high glucose [15]; which was in agreement with other studies demonstrating that IGFBP-3 is neuroprotective, anti-apoptotic, and anti-inflammatory after retinal injury [16]. Although IGFBP-3 was found to have protective role in retinal vessels, a paucity of information exists regarding the mechanism of action of IGFBP-3.

The structure of IGFBP-3 contains three distinct domains: a conserved N-terminal domain, a high variable mid-region, and a conserved C-terminal domain, as well as additional subdomains or functional motifs that may contribute to IGFBP-3 actions [17]. It has been found that binding between IGFBP-3 and cell surface proteins is specific and involves an interaction with glycosaminoglycans [18], located in the mid-region of IGFBP-3 [19]. Recent evidence suggested that IGFBP-3 has been functioned as a ligand for serine/threonine kinase type V TGF- β receptor (TGF- β RV). Furthermore, TGF- β RV/IGFBP-3 interactions activate cell signal pathways to modulate cell growth [20, 21]. TGF- β RV was found to be identical in function to the IGFBP-3 receptor, which mediates the IGF-independent growth response on stimulation of IGFBP-3 in responsive cells [22]. TGF- β RV was also found to have the same structure as low-density lipoprotein receptor-related protein-1 (LRP-1) [23]. It was reported that LRP-1/TGF- β RV/IGFBP-3 receptor is required for the growth inhibitory response to IGFBP-3 and TGF- β_1 in H1299 human lung carcinoma cells [23].

In this study, we investigated the presence of the IGFBP-3 receptor in inhibiting cell death related to diabetic retinopathy. We report that a loss of IGFBP-3 receptor levels results in a reduction of IGFBP-3's anti-apoptotic actions on REC cell growth in high ambient glucose, as assessed through IGFBP-3 receptor siRNA transfection.

Materials and methods

Reagents

Total and phosphorylated Akt (Ser 473), Cytochrome C, Bax, and Bcl-xL antibodies and a cleaved caspase 3 ELISA were purchased from Cell Signaling (Danvers, MA). Protein A/G PLUS-Agarose beads, IGFBP-3 antibodies (antimouse), IGFBP-3 receptor antibodies and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IGFBP-3 antibodies (anti-rabbit) were bought from Upstate (Lake Placid, NY). DyLight 594 goat antirabbit IgG antibodies were bought from Vector labs (Burlingame, CA). Human IGFBP-3 immunoassay ELISA kit was purchased from R & D Systems (Minneapolis, MN). Human LRP-1 siRNA and non-targeting siRNA # 1 were purchased from Dharmacon RNAi Technologies (Chicago, IL). FITC-goat anti-mouse IgG (H + L) antibodies, RNAlmax and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Secondary anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Promega (Madison, WI). ECL for immunoblot development and signal detection was purchased from Amersham Biosciences (Piscataway, NJ, USA). IGFBP-3 NB plasmid DNA is a gift from Dr. Maria B. Grant (University of Florida). Cell death ELISA kit was bought from Roche (Mannheim, Germany).

Cell culture

Primary human retinal microvascular endothelial cells (REC) were acquired from Cell System Corporation (CSC, Kirkland, Washington). Cells were grown in M131 medium containing microvascular growth supplements (Invitrogen) (MVGS), 10 µg/µl gentamycin and 0.25 µg/µl amphotericin B. In the high glucose condition, cells were transferred to high glucose (25 mM) (Cell Systems) medium, supplemented with MVGS and antibiotics for 3 days. To test whether osmolarity is a factor in the changes studied, we treated some cells with M131 medium containing 25 mM mannitol, supplemented with MVGS and antibiotics for 3 days. Only primary cells within passages 6 were used. Cells were quiesced by incubating in high or normal glucose medium without growth factors for 24 h and used to perform the experiments unless otherwise indicated.

Transfection of siRNA and plasmid DNA

ON-TARGETplus SMARTpool, human LRP-1 siRNA were purchased from Dharmacon, Inc. We used 4 sets of siRNA target sequences of GCGAAGGCAUUGUGU GUUC, GCACCAUUCUCAAGAGUAU, GCGCAUCGA UCUUCACAAA and GAACAAACACACUGGCUAA in LRP-1. siCONTROL Non-targeting siRNA #1 (Dharma-con) was used as a nonspecific control. REC were transfected with siRNA at a final concentration of 20 nM using RNAiMAX transfection reagent according to the manufacturer's instructions. The cells were used for experiments 24 h after transfection. The cells were also transfected with IGFBP-3 NB plasmid DNA at 1 µg/ml using Lipofectamine

2000, according to the manufacturer's instructions. The cells were used for experiments 24 h after transfection.

ELISA analysis

An ELISA for IGFBP-3 levels was performed using an IGFBP-3 ELISA assay kit according to the manufacturer's instructions to evaluate the IGFBP-3 expression following transfection with IGFBP-3NB plasmid DNA under normal or high glucose conditions. Cleaved caspase 3 was detected by ELISA to evaluate cell apoptosis under specific conditions, as recommended by the manufacturer. Cell death ELISA analyses of whole cell pellets were used to quantify apoptosis. For all ELISA analyses, equal protein amounts were loaded into each well, allowing for comparisons using optical density (O.D.).

Western blot analysis

After appropriate treatments and rinsing with cold phosphate-buffered saline, REC were scraped into lysis buffer containing the protease and phosphatase inhibitors. Equal amounts of protein from the cell extracts were separated on the pre-cast Tris-glycine gel (Invitrogen, Carlsbad, CA), blotted onto a nitrocellulose membrane. After blocking in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1 % Tween 20) containing 5 % (w/v) BSA, the membrane was treated with anti-IGFBP-3, IGFBP-3 receptor, Akt, pAkt, Bcl-xL, Bax, and Cytochrome C antibodies (all antibodies were used at 1:500 dilution) followed by incubation with horseradish peroxidase labeled secondary antibodies. The antigen-antibody complexes were detected using chemiluminescence reagent kit (Thermo Scientific).

Immunoprecipitation—After rinsing with phosphate-buffered saline (PBS), cells were lysed by freeze-thawing in lysis buffer containing the protease and phosphatase inhibitors for 20 min on ice. The cells were transferred into 1.5 ml Eppendorf tubes and cleared by centrifugation at 12,000 rpm for 20 min at 4 °C. The cells containing an equal amount of protein from control and each treatment were incubated with appropriate antibodies overnight at 4 °C at which time Protein A/G PLUS-agarose beads were added and incubation continued for 2 h with gentle rocking. The beads were washed three times with lysis buffer and once with PBS and the immunocomplexes were released by heating in Laemmli sample buffer and analyzed by Western blotting for the indicated molecules using their specific antibodies.

Immunofluorescence staining—REC were grown on chamber slides. Once confluent, cells were transfected with IGFBP-3 plasmid DNA for 24 h. The cells were then washed with PBS and fixed with 4 % paraformaldehyde for 1 h. After blocking in normal goat serum, the cell slides were incubated with goat anti-mouse IGFBP-3 receptor antibodies (1:100) and goat anti-rabbit IGFBP-3 receptor antibodies (1:100) in a humidified chamber overnight at 4 °C, followed by incubation with DyLight 594 goat anti-rabbit IgG antibodies (1:500) and FITC-goat anti-mouse IgG (H + L) antibodies (1:500). Slides were mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Fluorescence images were captured using a confocal microscope.

Statistics

All the experiments were repeated three times, and the data are presented as mean $\pm$ SEM. Data was analyzed by a non-parametric Kruskal-Wallis 1-way ANOVA, followed by a Dunn's test with p values <0.05 considered statistically significant. In the case of Western blotting, one representative blot is shown. The control was normalized to 1 and the treatment was compared with control by fold change.

Results

High glucose induced REC cell death compared with normal glucose or high osmolar condition

RECs were cultured in normal glucose (5 mM), high osmolar (25 mM mannitol) or high glucose (25 mM) for 3 days and cell extracts were test for cell apoptosis. From the result of the cell death ELISA (A) or cleaved caspase 3 ELISA (B), we found that high glucose induced cell apoptosis, while samples cultured in high osmolarity were not significantly different compared to normal glucose samples (Fig. 1).

IGFBP-3 inhibited REC cell death in high ambient glucose in a dose-range manner

To determine the optimal dose for IGFBP-3's inhibition of REC cell death, REC were transfected with IGFBP-3 NB plasmid DNA with dose range from 0.5 to 1.0 $\mu\text{g/ml}$ for 24 h in high ambient glucose. Results of the cell death ELISA and cleaved caspase 3 ELISA, shows that IGFBP-3 decreases cell death in high ambient glucose maximally at the 1 $\mu\text{g/ml}$ dose (Fig. 2).

IGFBP-3 induced IGFBP-3 receptor expression in high ambient glucose

It was recently reported that LRP-1/IGFBP-3 receptor is required for the growth response to IGFBP-3 [23]. Based on our hypothesis, we would expect increased IGFBP-3 receptor expression compared to untreated REC after IGFBP-3NB transfection. Our results showed there was an increased IGFBP-3 overexpression after IGFBP-3 transfection (Fig. 3a) and also IGFBP-3 NB plasmid increased IGFBP-3 receptor expression (Fig. 3b). Additionally, confocal imaging (Fig. 3c) of REC transfected with IGFBP-3 plasmid DNA showed co-localized expression of both IGFBP-3 receptor and IGFBP-3, consistent with the results from Western blot and ELISA analyses.

IGFBP-3 binds to IGFBP-3 receptor in high ambient glucose

To further test whether IGFBP-3 and its receptor binding is required for the regulation of cell death, REC were transfected with IGFBP-3 NB plasmid DNA and cell extracts were prepared and analyzed for coimmunoprecipitation. In Fig. 4a, cell extracts were first incubated with anti-IGFBP-3 antibodies and then re-probed with IGFBP-3 receptor or IGFBP-3 antibodies. Data revealed cells transfected with IGFBP-3 NB plasmid DNA showed overexpression of IGFBP-3 and a high level of IGFBP-3/IGFBP-3R binding. In Fig. 4b, cell extracts were first incubated with anti-IGFBP-3 receptor antibodies and then re-probed with IGFBP-3 or IGFBP-3 receptor antibodies. It also showed a high level of IGFBP-3 and its receptor binding and overexpression of IGFBP-3 receptor (Fig. 4b).

Coimmunoprecipitation assays revealed that the IGFBP-3 receptor forms a complex with IGFBP-3 in response to IGFBP-3 plasmid DNA transfection.

IGFBP-3 inhibited REC cell death in high ambient glucose was dependent on IGFBP-3 receptor

We and others have reported that IGFBP-3 can inhibit REC apoptosis [15]. In order to ascertain whether this occurs through activation of the IGFBP-3 receptor, we transfected IGFBP-3 receptor siRNA to determine its role in IGFBP-3 protection of cell death (Fig. 5). The results showed that IGFBP-3 receptor is required for IGFBP-3 to inhibit REC cell death in high ambient glucose.

Activation of IGFBP-3 receptor prevented REC apoptosis through activation of anti-apoptotic factors and down-regulation of pro-apoptotic factors

Analyses of key anti-apoptotic and pro-apoptotic factors demonstrated that IGFBP-3 receptor regulates cell death through increasing levels of Akt and Bcl-xL, while decreasing Cytochrome C, Bax and cleaved caspase 3 levels (Fig. 6). These results are the first to demonstrate that IGFBP-3 binds its receptor to regulate apoptotic proteins.

Discussion

IGFBP-3 has been shown to have IGF/IGF-1R-independent biological actions on the cell growth, proliferation and angiogenesis in diabetes [15, 24–27]. There has been intensive study on identifying molecular and cellular mechanisms for effects of IGFBP-3 in diabetic retinopathy both in vitro and in vivo. It was reported that IGFBP-3 and retinoid X receptor interaction can induce cancer cells apoptosis [28–30]; IGFBP-3 can bind to caveolin-1 and beta 1-integrins to enhance breast epithelial cell aggregation [31], and IGFBP-3 can bind transferrin to block smooth muscle cell proliferation [32]. Although these studies suggested that IGFBP-3 could mediate cell growth, the significance of the role of the binding partners remained unknown. Recently it was reported that T β R-V, which has the identical structure to LRP-1, served as the IGFBP-3 receptor in cancer cells and smooth muscle cells [22, 33, 34]. β R-V is a non-proteoglycan TGF- β receptor, co-localizes with TGF- β receptors (type I, II, and III) and mediates growth activity in most cell types. LRP-1 is known to be involved in cellular catabolism, plasma clearance of LRP-1 ligands and mediates intracellular signaling [22]. Since the LRP-1/IGFBP-3 receptor had not been identified in the retina, our goal was to define a function for the IGFBP-3 receptor in REC.

We now provide evidence that IGFBP-3 inhibition of REC apoptosis is mediated by the IGFBP-3 receptor. The molecular mechanism by which IGFBP-3 receptor mediates cell growth is largely unknown. In this work, we provide data to suggest that IGFBP-3 receptor is directly involved in IGFBP-3 functions in cell apoptosis. (1) REC transfected with IGFBP-3 plasmid DNA induced IGFBP-3 receptor expression; (2) IGFBP-3 inhibited REC apoptosis in a dose response pattern, which was blocked by IGFBP-3 receptor siRNA; and (3) IGFBP-3 and IGFBP-3 receptor bind each other tightly to form a complex by immunofluorescence staining and immunoprecipitation analyses. Based on our results and

previous publications, we propose that IGFBP-3 binds its receptor to prevent apoptosis of retinal endothelial cells [22, 33, 34].

To clarify the detailed mechanisms regarding to IGFBP-3 and its receptor in cell apoptosis, we used retinal endothelial cells in culture and investigated changes in DNA fragmentation and apoptosis markers when cells were transfected with IGFBP-3 plasmid DNA. To investigate whether that IGFBP-3 inhibit apoptosis of REC is IGFBP-3 receptor-dependent, we transfected IGFBP-3 receptor siRNA into REC. These data suggest that IGFBP-3 can decrease the apoptotic markers (Bax and Cytochrome C) and increase the anti-apoptotic proteins (Bcl-xL and Akt) compared with cells cultured in high glucose conditions. Additionally, the ability for IGFBP-3 to inhibit REC apoptosis is IGFBP-3 receptor dependent. The mechanisms by which IGFBP-3/IGFBP-3 receptor can be anti-apoptotic for REC are unknown. Our results suggest that they could regulate the Bcl-2 family. Bcl-xL, a pro-survival member, can prevent the release of Cytochrome C from mitochondria [35]. Bax can mediate caspase-independent cell death via channel-forming activities, which can also promote mitochondrial disruption [35]. The exact mechanism of how IGFBP-3/IGFBP-3 receptor can regulate REC apoptosis and mito-chondrial changes is a focus of future studies.

Endothelial cell dysfunction plays a prominent role in the pathophysiological complications of diabetic retinopathy. The findings that IGFBP-3/IGFBP-3 receptor inhibited REC cell apoptosis has many potential implications. IGFBP-3 receptor may regulate in the acceleration of diabetic mellitus. It is hoped further studies to regulate IGFBP systems will be useful in halting the progression of diabetic retinopathy.

Acknowledgments

Supported by National Eye Institute Vision Grant R01EY022045 (JJS); Juvenile Diabetes Research Foundation Grant (2-2011-597 to JJS); Oxnard Foundation (JJS); Research to Prevent Blindness Award (PI:Barrett Haik); and NEI Vision Core Grant: PHS 3P30 EY013080 (PI: Dianna Johnson).

References

1. Yamada PM, Lee KW. Perspectives in mammalian IGFBP-3 biology: local vs. systemic action. *Am J Physiol Cell Physiol.* 2009; 296:C954–976. [PubMed: 19279229]
2. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev.* 2002; 23:824–854. [PubMed: 12466191]
3. Perks C, Holly J. Actions of IGFBP on epithelial cancer cells: potential for new therapeutic targets. *Horm Metab Res.* 2003; 35:828–835. [PubMed: 14710365]
4. Schedlich LJ, Graham LD, O'Han MK, Muthukaruppan A, Yan X, Firth SM, et al. Molecular basis of the interaction between IGFBP-3 and retinoid X receptor: role in modulation of RAR-signaling. *Arch Biochem Biophys.* 2007; 465:359–369. [PubMed: 17644060]
5. Lee YC, Jogie-Brahim S, Lee DY, Han J, Harada A, Murphy LJ, et al. Insulin-like growth factor-binding protein-3 (IGFBP-3) blocks the effects of asthma by negatively regulating NF-kappaB signaling through IGFBP-3R-mediated activation of caspases. *J Biol Chem.* 2011; 286:17898–17909. [PubMed: 21383009]
6. Ikezoe T, Tanosaki S, Krug U, Liu B, Cohen P, Taguchi H, et al. Insulin-like growth factor binding protein-3 antagonizes the effects of retinoids in myeloid leukemia cells. *Blood.* 2004; 104:237–242. [PubMed: 15026318]
7. Duschek EJ, de Valk-de Roo GW, Gooren L, Netelenbos C. Effects of conjugated equine estrogen vs. raloxifene on serum insulin-like growth factor-i and insulin-like growth factor binding protein-3:

- a 2-year, double-blind, placebo-controlled study. *Fertil Steril*. 2004; 82:384–390. [PubMed: 15302288]
8. Grimberg A, Coleman CM, Burns TF, Himelstein BP, Koch CJ, Cohen P, et al. p53-Dependent and p53-independent induction of insulin-like growth factor binding protein-3 by deoxyribonucleic acid damage and hypoxia. *J Clin Endocrinol Metab*. 2005; 90:3568–3574. [PubMed: 15769996]
 9. Am HZ, Collard TJ, Malik K, Hicks DJ, Paraskeva C, Williams AC. Induction of apoptosis by the 16-kDa amino-terminal fragment of the insulin-like growth factor binding protein 3 in human colonic carcinoma cells. *Int J Oncol*. 2006; 29:1279–1286. [PubMed: 17016662]
 10. Lofqvist C, Chen J, Connor KM, Smith AC, Aderman CM, Liu N, et al. IGFBP3 suppresses retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth. *Proc Natl Acad Sci USA*. 2007; 104:10589–10594. [PubMed: 17567756]
 11. Schmid MC, Bisoffi M, Wetterwald A, Gautschi E, Thalmann GN, Mitola S, et al. Insulin-like growth factor binding protein-3 is overexpressed in endothelial cells of mouse breast tumor vessels. *Int J Cancer*. 2003; 103:577–586. [PubMed: 12494464]
 12. Lofqvist C, Willett KL, Aspegren O, Smith AC, Aderman CM, Connor KM, et al. Quantification and localization of the IGF/insulin system expression in retinal blood vessels and neurons during oxygen-induced retinopathy in mice. *Invest Ophthalmol Vis Sci*. 2009; 50:1831–1837. [PubMed: 18997086]
 13. Janssen JA, Jacobs ML, Derkx FH, Weber RF, van der Lely AJ, Lamberts SW. Free and total insulin-like growth factor I (IGF-I), IGF-binding protein-1 (IGFBP-1), and IGFBP-3 and their relationships to the presence of diabetic retinopathy and glomerular hyperfiltration in insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab*. 1997; 82:2809–2815. [PubMed: 9284701]
 14. Regan FM, Williams RM, McDonald A, Umpleby AM, Acerini CL, O'Rahilly S, et al. Treatment with recombinant human insulin-like growth factor (rhIGF)-I/rhIGF binding protein-3 complex improves metabolic control in subjects with severe insulin resistance. *J Clin Endocrinol Metab*. 2010; 95:2113–2122. [PubMed: 20233784]
 15. Zhang Q, Guy K, Pagadala J, Jiang Y, Walker RJ, Liu L, et al. Compound 49b prevents diabetes-induced apoptosis through increased IGFBP-3 levels. *Invest Ophthalmol Vis Sci*. 2012; 53:3004–3013. [PubMed: 22467575]
 16. Kielczewski JL, Hu P, Shaw LC, Li Calzi S, Mames RN, Gardiner TA, et al. Novel protective properties of IGFBP-3 result in enhanced pericyte ensheathment, reduced microglial activation, increased microglial apoptosis, and neuronal protection after ischemic retinal injury. *Am J Pathol*. 2011; 178:1517–1528. [PubMed: 21435441]
 17. Yan X, Forbes BE, McNeil KA, Baxter RC, Firth SM. Role of N- and C-terminal residues of insulin-like growth factor (IGF)-binding protein-3 in regulating IGF complex formation and receptor activation. *J Biol Chem*. 2004; 279:53232–53240. [PubMed: 15485880]
 18. Oh Y, Muller HL, Pham H, Rosenfeld RG. Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. *J Biol Chem*. 1993; 268:26045–26048. [PubMed: 7504671]
 19. Jogie-Brahim S, Feldman D, Oh Y. Unraveling insulin-like growth factor binding protein-3 actions in human disease. *Endocr Rev*. 2009; 30:417–437. [PubMed: 19477944]
 20. Leal SM, Liu Q, Huang SS, Huang JS. The type V transforming growth factor beta receptor is the putative insulin-like growth factor-binding protein 3 receptor. *J Biol Chem*. 1997; 272:20572–20576. [PubMed: 9252371]
 21. Leal SM, Huang SS, Huang JS. Interactions of high affinity insulin-like growth factor-binding proteins with the type V transforming growth factor-beta receptor in mink lung epithelial cells. *J Biol Chem*. 1999; 274:6711–6717. [PubMed: 10037769]
 22. Huang SS, Leal SM, Chen CL, Liu IH, Huang JS. Identification of insulin receptor substrate proteins as key molecules for the TbetaR-V/LRP-1-mediated growth inhibitory signaling cascade in epithelial and myeloid cells. *FASEB J*. 2004; 18:1719–1721. [PubMed: 15371331]
 23. Huang SS, Ling TY, Tseng WF, Huang YH, Tang FM, Leal SM, et al. Cellular growth inhibition by IGFBP-3 and TGF-beta1 requires LRP-1. *FASEB J*. 2003; 17:2068–2081. [PubMed: 14597676]

24. Natsuzaka M, Naganuma S, Kagawa S, Ohashi S, Ahmadi A, Subramanian H, et al. Hypoxia induces IGFBP3 in esophageal squamous cancer cells through HIF-1 α -mediated mRNA transcription and continuous protein synthesis. *FASEB J.* 2012; 26:2620–2630. [PubMed: 22415309]
25. King JL, Guidry C. Vitreous IGFBP-3 effects on Muller cell proliferation and tractional force generation. *Invest Ophthalmol Vis Sci.* 2012; 53:93–99. [PubMed: 22110068]
26. Gribben L, Baxter RC, Marsh DJ. Insulin-like growth factor binding protein-3 inhibits migration of endometrial cancer cells. *Cancer Lett.* 2012; 317:41–48. [PubMed: 22085490]
27. Kim JH, Choi DS, Lee OH, Oh SH, Lippman SM, Lee HY. Antiangiogenic antitumor activities of IGFBP-3 are mediated by IGF-independent suppression of Erk1/2 activation and Egr-1-mediated transcriptional events. *Blood.* 2011; 118:2622–2631. [PubMed: 21551235]
28. Liu B, Lee HY, Weinzimer SA, Powell DR, Clifford JL, Kurie JM, et al. Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor- α regulate transcriptional signaling and apoptosis. *J Biol Chem.* 2000; 275:33607–33613. [PubMed: 10874028]
29. Liu B, Lee KW, Li H, Ma L, Lin GL, Chandraratna RA, et al. Combination therapy of insulin-like growth factor binding protein-3 and retinoid X receptor ligands synergize on prostate cancer cell apoptosis in vitro and in vivo. *Clin Cancer Res.* 2005; 11:4851–4856. [PubMed: 16000583]
30. Lee KW, Ma L, Yan X, Liu B, Zhang XK, Cohen P. Rapid apoptosis induction by IGFBP-3 involves an insulin-like growth factor-independent nucleomitochondrial translocation of RXR α /Nur77. *J Biol Chem.* 2005; 280:16942–16948. [PubMed: 15731112]
31. Burrows C, Holly JM, Laurence NJ, Vernon EG, Carter JV, Clark MA, et al. Insulin-like growth factor binding protein 3 has opposing actions on malignant and nonmalignant breast epithelial cells that are each reversible and dependent upon cholesterol-stabilized integrin receptor complexes. *Endocrinology.* 2006; 147:3484–3500. [PubMed: 16614079]
32. Weinzimer SA, Gibson TB, Collett-Solberg PF, Khare A, Liu B, Cohen P. Transferrin is an insulin-like growth factor-binding protein-3 binding protein. *J Clin Endocrinol Metab.* 2001; 86:1806–1813. [PubMed: 11297622]
33. Kuemmerle JF, Murthy KS, Bowers JG. IGFBP-3 activates TGF- β receptors and directly inhibits growth in human intestinal smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol.* 2004; 287:G795–802. [PubMed: 15178549]
34. Ingermann AR, Yang YF, Han J, Mikami A, Garza AE, Mohanraj L, et al. Identification of a novel cell death receptor mediating IGFBP-3-induced anti-tumor effects in breast and prostate cancer. *J Biol Chem.* 2010; 285:30233–30246. [PubMed: 20353938]
35. Martinou JC, Youle RJ. Mitochondria in apoptosis: bcl-2 family members and mitochondrial dynamics. *Dev Cell.* 2011; 21:92–101. [PubMed: 21763611]

Fig. 1.

High glucose inhibited REC cell death compared to a normal ambient glucose or a high osmolarity control. *Bar graph* of DNA fragmentation levels measured by the Roche Cell Death ELISA kit (**a**) and cleaved caspase 3 levels by cleaved caspase 3 ELISA kit (**b**) in REC following incubation in 5 mM glucose, 25 mM glucose and 25 mM mannitol. * $p < 0.05$ versus 5 mM glucose control (N = 4)

Fig. 2. IGFBP-3 inhibited REC cell death in a dose-dependent manner in REC in high ambient glucose. Bar graph of DNA fragmentation levels measured by the Roche Cell Death ELISA kit (a) and cleaved caspase 3 levels by cleaved caspase 3 ELISA kit (b) in REC following transfection with different dose of IGFBP-3 plasmid DNA. * $p < 0.05$ versus control (N = 4)

Fig. 3. IGFBP-3 overexpression stimulated IGFBP-3 receptor expression in high glucose medium. **a** ELISA analysis of IGFBP-3 levels in REC transfected with control or IGFBP-3 NB plasmid DNA for 24 h in medium containing normal glucose (NG-5 mM) or high glucose (HG-25 mM) medium. **b** Expression of IGFBP-3 receptor after IGFBP-3NB plasmid DNA transfection in REC in normal and high glucose. * $p < 0.05$ versus NG control plasmid DNA transfection. # $p < 0.05$ versus HG control plasmid DNA transfection (N = 3). **c** Representative images of REC with IGFBP-3 plasmid DNA transfection: (left, yellow) merge, (middle, red) IGFBP-3 receptor, (right, green) IGFBP-3. Scale bars of 16 μm are present in each image (Color figure online)

Fig. 4.

Coimmunoprecipitation of IGFBP-3 and IGFBP-3 receptor from REC transiently transfected with IGFBP-3 plasmid DNA. **a** Proteins from REC transfected with IGFBP-3 plasmid DNA was immunoprecipitated with anti-IGFBP-3 antibodies. The immunoprecipitates were analyzed by Western blotting using anti-IGFBP-3 receptor or anti-IGFBP-3 antibodies. **b** All conditions were the same as **a** except proteins were immunoprecipitated with anti-IGFBP-3 receptor antibodies. * $p < 0.05$ versus NG control plasmid, # $p < 0.05$ versus HG control plasmid (N = 3)

Fig. 5. Representative of IGFBP-3 receptor blot and *bar graph* of IGFBP-3 receptor levels in REC transfected with scrambled or IGFBP-3 receptor siRNA for 24 h. $*p < 0.05$ versus scrambled siRNA, $^{\#}p < 0.05$ versus HG scrambled siRNA (N = 3)

Fig. 6. IGFBP-3 decreased cell death in REC in high ambient glucose is IGFBP-3 receptor dependent. **a–b.** Bar graph of DNA fragmentation (**a**) and cleaved caspase 3 (**b**) of REC in normal or high glucose transfected with IGFBP-3 receptor siRNA and IGFBP-3NB plasmid. * $p < 0.05$ versus NG control plasmid, # $p < 0.05$ versus HG control plasmid, \$ $p < 0.05$ versus HG IGFBP-3 NB plasmid (N = 8). **c–f.** Bar graphs and representative blots for Cytochrome C(**f**), Bax (**e**), Bcl-xL(**d**), and Akt (**c**) for cell cultures in normal glucose (NG) or high glucose (HG) and co-transfected with IGFBP-3 NB plasmid and IGFBP-3 receptor siRNA. * $p < 0.05$ versus NG control plasmid, # $p < 0.05$ versus HG control plasmid, \$ $p < 0.05$ versus HG IGFBP-3 NB plasmid (N = 3)