THE ROLE OF THE IGF/INSULIN-IGFBP AXIS IN NORMAL PHYSIOLOGY AND DISEASE

EDITED BY: Claire Perks and Shin-Ichiro Takahashi
PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-142-5
DOI 10.3389/978-2-88976-142-5

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THE ROLE OF THE IGF/INSULIN-IGFBP AXIS IN NORMAL PHYSIOLOGY AND DISEASE

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Citation: Perks, C., Takahashi, S.-I., eds. (2022). The Role of the IGF/Insulin-IGFBP Axis in Normal Physiology and Disease. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-142-5

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frontiers | Frontiers in Endocrinology

EDITORIAL
published: 21 April 2022
doi: 10.3389/fendo.2022.892140

Editorial: The Role of the IGF/Insulin-IGFBP Axis in Normal Physiology and Disease

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Keywords: IGF, IGFBPs, insulin, ageing, cancer, GRC

Editorial on the Research Topic

The Role of the IGF/Insulin-IGFBP Axis in Normal Physiology and Disease

The 2019 Gordon Research Conference (GRC) on the insulin-like growth factor (IGF) and Insulin System in Physiology and Disease was held from March 10th to 15th in 2019. In that meeting, we focused on "The Impact of IGF and Insulin on Life-Long Health". We discussed cutting-edge research on the fundamental roles of IGF and insulin in normal physiology and diseases particularly related to aging, cancer, and metabolic disorders. IGFs and insulin are conserved throughout evolution to mediate the effects of nutrition on growth, metabolism, and development, and hence play a significant role in health and disease over the lifespan. They modulate diverse aspects of cell function such as proliferation, differentiation, survival, and metabolism of most physiological systems in the body. The IGF family of ligands, receptors, and IGF binding proteins are frequently affected in many pathological conditions, such as growth failure, diabetes, cancer, and degenerative diseases, and therefore have become attractive therapeutic targets. This conference encompassed ground-breaking information regarding critical characteristics of the biology of the IGF/insulin family in both normal physiology and pathological states highlighting innovative methodologies and novel interactions (*e.g.*, stem cell biology and the microbiome). We selected eight exciting topics presented at the GRC for this special issue on "The Role of the IGF/Insulin-IGFBP Axis in Normal Physiology and Disease" The IGFBPs are frequently dysregulated in pathological conditions and Duan and Allard, discussed what is currently known about IGFBP-5 in normal physiology and human disease. They concluded that IGFBP-5 is a multifunctional protein that can act as a molecular switch to regulate IGF signaling conditionally. Therapy resistance is a major problem in cancer treatment and Zheng et al., discovered that IGFBP-1 plays a significant role in resistance to a selective estrogen receptor modulator and antagonist for estrogen receptor alpha (ERα) in breast tissue, called Tamoxifen. IGFBPs can be post-translationally modified, for example *via* proteolytic cleavage and this also has implications for disease. Hoeflich et al., found that reduced fragmentation of IGFBPs and concomitant reduction of IGF-II to IGFBP ratios modulated the bioactivity of IGF-II in cerebrospinal fluid during repeated intrathecal triamcinolone acetonide administration in multiple sclerosis patients, which may have relevance for treatment. In addition, Hjortebjerg et al., showed that pregnancy-associated plasma protein-A (PAPP-A) and its homolog PAPP-A2 which are reported as IGFBP proteases are enzymes that modulate the availability and mitogenic activity of IGF-I. Collectively, the data show that PAPP-A2, but not PAPP-A, is elevated in patients with lung cancer and is associated with mortality. This novel role of PAPP-A2 in cancer warrants further functional studies as well as validation in external cohorts. As for signal transduction

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Edited and reviewed by:
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Specialty section:
This article was submitted to Molecular and Structural Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *08 March 2022*
Accepted: *17 March 2022*
Published: *21 April 2022*

Citation:
Takahashi S-I and Perks CM (2022) Editorial: The Role of the IGF/Insulin-IGFBP Axis in Normal Physiology and Disease. Front. Endocrinol. 13:892140. doi: 10.3389/fendo.2022.892140

of insulin-like peptides, the mini-review by Rieger and O'Connor, introduced data showing that IGF-I receptor endocytosis and trafficking to specific subcellular locations can define specific signaling responses that are important for key biological processes in normal cells and cancer cells. Once internalized, the IGF-I receptor may be recycled, degraded, or translocated to the intracellular membrane compartments of the Golgi apparatus or the nucleus leading to different outcomes. Okino et al., showed that the high levels of insulin receptor substrate (IRS)-1 in myoblasts induces their elimination from the cell layer due to abnormal sustainment of IGF-I receptor activation. This cell competition plays a vital role in myotube formation. The mini-review by Barker et al., presents a brief overview examining aspects of IGFs and the PI3K/Akt pathway in two apparently unconnected diseases: Alzheimer's dementia and cancer. Although these disease states appear to be opposed, the same vital molecules are controlling pathology and, differential targeting of therapeutics, may benefit both. Finally, Stuard et al., provided the latest update on the function of IGF and related proteins in corneal development, during wound healing, and in the pathophysiology of disease and highlighted key areas of research that are necessary for future studies. From *C. elegans* to rhesus monkeys, it has been reported that suppression of insulin-like activity is associated with an increased life span. Together with other reports from this GRC, excessive induction of insulin-like activity can lead to cancer, and excessive attenuation leads to various diseases that are also problematic in an aging society, such as Alzheimers. These results clearly demonstrate the importance of regulating insulin-like activity to an appropriate range to maintain a lifetime of good health. This regulation is accomplished through ligand production, interaction with binding proteins, receptor expression, and signaling. The future mission of this research area is to elucidate how abnormalities in molecular signalling pathways utilized by the IGF axis correlate with the phenotype associated with pathological conditions, to develop preventive and therapeutic interventions, leading to higher quality resource animals and an increase in healthy life expectancy in humans. The IGFs are clearly relevant to all life phenomena and as such have attracted many researchers to the field from different backgrounds, highlighted by the varied and diverse presentations covering both normal physiology and disease. An IGF focus within a cross-disciplinary approach yields exciting, novel and groundbreaking discoveries that were presented at the GRC, and the participants all shared in the excitement. We are delighted for the broader community to provide a taste of this GRC in this special issue. The next GRC on the IGF and Insulin System in Physiology and Disease will be held in March 2023. We look forward to seeing you all there.

AUTHOR CONTRIBUTIONS

ST and CP co-wrote the editorial. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by Grants-in-Aid for Scientific Research (A) #18H03972, Advanced Research Networks from the Japan Society for the Promotion of Science (JSPS) and R&D matching funds on the field for Knowledge Integration and innovation and Moonshot Agriculture, Forestry and Fisheries Research and Development Program from Bio-oriented Technology Research Advancement Institution (BRAIN) to ST.

ACKNOWLEDGMENTS

We would like to thank Dr. Susan Hall (The University of North Carolina at Chapel Hill) for various scientific suggestions.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 February 2020
doi: 10.3389/fendo.2020.00096

Myoblasts With Higher IRS-1 Levels Are Eliminated From the Normal Cell Layer During Differentiation

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Insulin receptor substrate (IRS)-1 is a major substrate of insulin-like growth factor (IGF)-I receptors. It is well-known that IGF-I and II play essential roles in myogenesis progression. Herein, we report an unexpected phenomenon that IRS-1-overexpressing L6 myoblasts are eliminated from normal cell layers at the beginning of differentiation. Initially, the IRS protein level and apoptosis were examined during myogenic differentiation in L6 myoblasts. We found that the IRS-1 protein level decreased, whereas active caspase 3 increased around 1 day after induction of differentiation. The addition of a pan-caspase inhibitor, Z-VAD-FMK, inhibited differentiation-induced suppression of the IRS-1 protein level. Apoptosis was not enhanced in L6 myoblasts stably expressing high levels of IRS-1 (L6-IRS-1). However, when L6-IRS-1 was cultured with control cells (L6-mock), we observed that L6-IRS-1 was eliminated from the cell layer. We have recently reported that, in L6-IRS-1, internalization of the IGF-I receptor was delayed and IGF signal activation was sustained for a longer period than in L6-mock. When cells stably expressing IRS-1 3YA mutant, which could not maintain the IGF signals, were cultured with normal cells, elimination from the cell layer was not detected. These data suggested that the high level of IRS-1 in myoblasts induces elimination from the cell layer due to abnormal sustainment of IGF-I receptor activation.

Keywords: insulin-like growth factor (IGF)-I, L6 myoblasts, myogenesis, insulin receptor substrate (IRS)-1, cell competition

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Edited by:
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *12 November 2019*
Accepted: *14 February 2020*
Published: *28 February 2020*

Citation:
Okino R, Usui A, Yoneyama Y, Takahashi S-I and Hakuno F (2020) Myoblasts With Higher IRS-1 Levels Are Eliminated From the Normal Cell Layer During Differentiation. Front. Endocrinol. 11:96. doi: 10.3389/fendo.2020.00096

INTRODUCTION

Myogenic differentiation is a tightly regulated complex process in which mononucleated myoblasts proliferate, express myogenic marker proteins (MyoD, myogenin, myosin heavy chain (MyHC), etc.), and fuse to form multinucleated myotubes. Matured myotubes convert into myofibers, which are capable of muscle contraction. These multiple processes of myogenic differentiation seem to depend on numerous pathways (1, 2). Extensive investigations using myoblast cell lines and tissues revealed that several extracellular growth factors modulate myogenic differentiation (3–5). Many papers have shown that insulin and insulin-like growth factors (IGFs) stimulate myoblast differentiation and are required for skeletal muscle development (6–9).

IGF-I and IGF-II are anabolic hormones with structures similar to that of proinsulin. IGFs are revealed to possess various bioactivities, including the induction of cell proliferation,

differentiation, and survival of target tissues. Generally, by binding to their specific receptors on the plasma membrane, IGFs activate intrinsic tyrosine kinase activity. The activated receptor phosphorylates several substrates, including insulin receptor substrates (IRSs). Phosphotyrosine residues in IRSs are recognized by several signaling molecules with an SH2 domain, resulting in activation of the phosphatidyl inositol 3-kinase-Akt pathway and Ras-mitogen activated protein kinase pathway. Activation of these pathways is shown to be required for the expression of various IGF bioactivities.

It is well-established that IGFs are required for myogenic differentiation. In particular, in serum-free medium, myogenic differentiation was blocked and IGF addition significantly enhanced the creatine kinase level (10). Thus, IGF has an essential role in myogenic differentiation. However, it is unclear whether the activation of downstream IGF signaling pathways is constantly required for myogenesis. For example, IRS-1 knockdown C2C12 myoblasts had defects in myogenesis (11). On the other hand, we previously reported that IRS-1 overexpression inhibited myogenic differentiation in L6 myoblasts through continuous Foxo1 inhibition that might cause repression of MyHC at the late stage of differentiation (12).

Recent reports demonstrated that apoptotic cells are necessary for the myogenic differentiation process. The phosphatidylserine receptor BAI1, which was previously linked to apoptotic cell recognition by phagocytes, promotes myoblast fusion. Blocking apoptosis during myogenic differentiation potently impaired this process; furthermore, returning apoptotic myoblasts to this system restored fusion (13). On the other hand, endoplasmic reticulum (ER) stress signaling occurs during myoblast differentiation, and inhibition of ER stress signaling blocked apoptosis and myoblast differentiation. Moreover, increased ER stress enhanced differentiation-associated apoptosis of myoblasts (14). Thus, apoptosis is required for myogenic differentiation. However, the types of cells that selectively undergo apoptosis or differentiate into myotubes during myogenic differentiation remain unknown.

This study was undertaken to evaluate the mechanism of IGF signal regulation of myoblast proliferation and apoptosis during myogenic differentiation. We found that cells expressing high IRS-1 levels are eliminated from the normal cell layer and undergo apoptosis upon culturing with normal cells due to sustained IGF signal activation.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Sigma Aldrich (St. Louis, MO, USA). Penicillin and streptomycin were obtained from Banyu Pharmaceutical Co. (Ibaraki, Japan). Z-VAD-FMK was obtained from BD Biosciences (New York, NY, USA).

Antibodies

Anti-IRS-2 (390761) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-IRS1 (06-248), anti-myosin heavy chain (05-716) and anti-p85 (06-195) antibodies were acquired from Millipore (Billerica, MA, USA). Anti-caspase 3 (#9662), anti-cleaved caspase 3 (#9661), and anti-Bax (#2772) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit (NA934) and anti-mouse IgG (NA931) antibodies were obtained from GE Healthcare (Pittsburgh, PA, USA). Antibodies were diluted according to the recommendations on their data sheets. Enhanced chemiluminescence (ECL) reagents were acquired from PerkinElmer Life Science (Boston, MA, USA). Alexa Fluor 488 or 594-conjugated secondary anti-mouse, anti-rabbit, or anti-rat IgG antibodies were obtained from Invitrogen (Carlsbad, CA, USA).

Cell Culture

L6 cells were maintained at 37°C in a humidified 5% CO_2-controlled atmosphere in DMEM supplemented with 10% FBS, 0.1% $NaHCO_3$, 50 IU/mL penicillin, and 50 μg/mL streptomycin. L6 cell differentiation was induced as previously described (12). Passage number of cells used in experiments was 8∼15. In each experiment, passage number of the cell lines are same. PLAT-E cells were cultured for retrovirus packaging as previously described (15).

Retrovirus Production and Generation of Stable Cell Lines

We generated the constructs of the pMX-neo vectors containing IRS-1 (pMX-GFP-IRS-1, pMX-mycIRS-1, and pMX-IRS-1-3YA) and pMX-puro vector containing GFP (pMX-GFP). Retrovirus production and transduction in L6 cells were performed as described previously (15). Briefly, PLAT-E cells (provided by T. Kitamura, The University of Tokyo, Tokyo, Japan) were transiently transfected with each pMX vector using polyethylenimine (PEI) reagent, and the media containing the retrovirus were collected. L6 cells were incubated with the virus-containing medium supplemented with 2 mg/L polybrene. Uninfected cells were removed by G418 or puromycin selection. Isolation of the stable L6 line was performed as described previously (12).

Cell Attachment Assay

Five million L6-mock cells were seeded on a 35 mm dish and cultured until confluent. Either L6-mycIRS-1-GFP or L6-GFP cells were then seeded on the L6-mock confluent cell layer or on a vacant dish. One day after the incubation, the cells were fixed, and the numbers of GFP-positive cells were counted. The cell layer attachment index (CLAI) was calculated as the number of GFP-positive cells attached on the cell layer divided by GFP-positive cells attached on the vacant dish.

Immunoblotting

Cells were lysed at 4°C with ice-cold lysis buffer (1% NP40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM

NaF, 10% glycerol, 20 μg/mL phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL pepstatin, 10 μg/mL leupeptin, 100 KIU/mL aprotinin, 1 mM Na_3VO_4, and 10 mg/mL *p*-nitrophenyl phosphate), or ice-cold RIPA buffer (50 mM Tris-HCl [pH 7.4], 15 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 20 μg/mL PMSF, 5 μg/mL pepstatin, 10 μg/mL leupeptin, 100 KIU/ml aprotinin, 1 mM Na_3VO_4, and 10 mg/mL *p*-nitrophenyl phosphate). Insoluble materials were removed by centrifugation at 15,000 × g for 10 min at 4°C, and the supernatant was prepared as a total cell lysate. Immunoblotting was performed as described previously (15).

Immunofluorescence Staining

For confocal microscopy analysis, L6 cells were grown on coverslips. The cells were fixed for 10 min at 25°C in prewarmed 4% paraformaldehyde in phosphate-buffered saline (PBS). After washing three times with PBS, cells were permeabilized with 0.25% Triton X-100 in PBS at 25°C for 10 min. The cells were washed three times with PBS and then blocked for 1 h at 4°C with bovine serum albumin (BSA) blocking buffer (3% BSA and 0.025% NaN_3 in PBS). Primary antibodies diluted in BSA blocking buffer were added overnight at 4°C. The samples were washed three times with PBS and incubated for 1 h at 25°C in a solution of Alexa Fluor-conjugated secondary antibodies diluted in BSA blocking buffer. The coverslips were mounted using Vectashield for visualization using a fluorescence microscope (KEYENCE, Tokyo, Japan) or confocal fluorescence microscope (OLYMPUS, Tokyo, Japan).

Statistical Analysis

Statistical analyses of data were performed using Stat View software (Abacus Concepts, Inc., Berkeley, CA, USA). Comparisons between two groups were analyzed by Student's *t*-test, and more than two groups were analyzed by ANOVA followed by Turkey's test. Differences were considered to be statistically significant at $P < 0.05$, as represented by *.

RESULTS

Protein Levels of IRS-1 and Cleaved Caspase 3 Were Dramatically Changed During Myogenic Differentiation of L6 Myoblasts

Differentiation of L6 myoblasts was induced by changing media from DMEM with 10% FBS to DMEM with 2% FBS. As shown in **Figure 1A**, we could confirm that expression of the myogenic marker protein myosin heavy chain increased 2 days after the induction of differentiation. Protein levels of IRS-1 or IRS-2 were examined by immunoblotting analysis. The IRS-2 protein level was not changed during differentiation induction, whereas that of IRS-1 decreased only 1 day after induction. Interestingly, the level of cleaved caspase 3, an apoptotic marker protein and active form of caspase 3, increased ∼0.75 day after differentiation induction; this indicated that apoptotic cells were generated, then IRS-1 protein was decreased. In addition, when the apoptosis inhibitor Z-VAD-FMK was added to the differentiation medium, the IRS-1 protein level did not decrease (**Figure 1B**). Since the IRS-1 protein level decreased just after apoptosis activation, we generated the hypothesis that cells highly expressing IRS-1 selectively undergo apoptosis.

To address whether IRS-1 overexpression enhances apoptosis, we infected L6 myoblasts with retroviruses expressing mock vector, GFP, or GFP-fused IRS-1 and isolated the stable cell lines L6-mock, L6-GFP, and L6-GFP-IRS-1. We could confirm that the GFP-IRS-1 expression level was high in L6-GFP-IRS-1 lines (**Figure 1C**). Caspase 3 activation was examined and found to be activated 1 day after inducing differentiation in L6-mock and L6-GFP control cells. However, in L6-GFP-IRS-1, caspase 3 was not activated (**Figure 1C**). Immunostaining analysis against cleaved caspase 3 (active caspase 3) also indicated that apoptosis was suppressed in L6-GFP-IRS-1 cells (**Figure 1D**). These data indicated that IRS-1 overexpression did not enhance apoptosis.

Cells Overexpressing IRS-1 Were Selectively Excluded When They Were Surrounded by Normal Cells

To examine the fate of cells overexpressing IRS-1 within a normal cell population, L6-GFP-IRS-1 or L6-GFP stable cell lines were mixed with normal L6 cells (L6-mock) at a ratio of 1:10. These cells were then cultured in 10% FBS medium until confluent. The mixture of the two cell lines was cultured in the differentiation medium for the indicated days. When L6-GFP was cultured with normal L6-mock, the number of GFP-positive cells (L6-GFP) increased at a similar ratio as that of the total cell number (**Figure 2A**). On the contrary, when L6-GFP-IRS-1 was cultured with L6-mock, the cell number of L6-GFP-IRS-1 decreased (**Figure 2A**). When L6-GFP was cultured with L6-mock, the ratio of GFP-positive cells remained unchanged until day 4 compared to day 0. However, when L6-GFP-IRS-1 was cultured with L6-mock, the ratio of GFP-positive cells decreased at day 4 compared to day 0 (**Figure 2B**). These data strongly suggested that L6-GFP-IRS-1 was selectively excluded from the cell layer.

Because protein degradation of IRS-1 is induced by the activation of the downstream IGF signal kinase mTORC1 (16–19), it is possible that the level of GFP-fused IRS-1 also degraded and the GFP signal diminished in response to IGF signal activation. To exclude this possibility, we generated stable cell lines expressing both mycIRS-1 and GFP independently (L6-mycIRS-1-GFP). At first Caspase 3 activation was examined in the single culture system. By the induction of differentiation, Caspase 3 activation was not enhanced also in L6-mycIRS-1-GFP (**Figure S1**). When L6-mycIRS-1-GFP was cultured with L6-mock at a ratio of 1:1, it was also selectively eliminated (**Figure 2C**). The ratio of L6-mycIRS-1-GFP cells decreased on day 2 compared to day 0, while the ratio of L6-GFP cells remained unchanged (**Figure 2D**). Moreover, growth rate of L6-mock, L6-GFP, or L6-mycIRS1-GFP was almost comparable (**Figure S2**). Although IRS-1 overexpression did neither enhance apoptosis (**Figures 1C,D**, **Figure S1**) nor suppress proliferation rate (**Figure S2**), the cell number of L6-mycIRS-1-GFP are selectively decreased only when these cells are cultured with normal cells (**Figure 2**). These data strongly suggested that

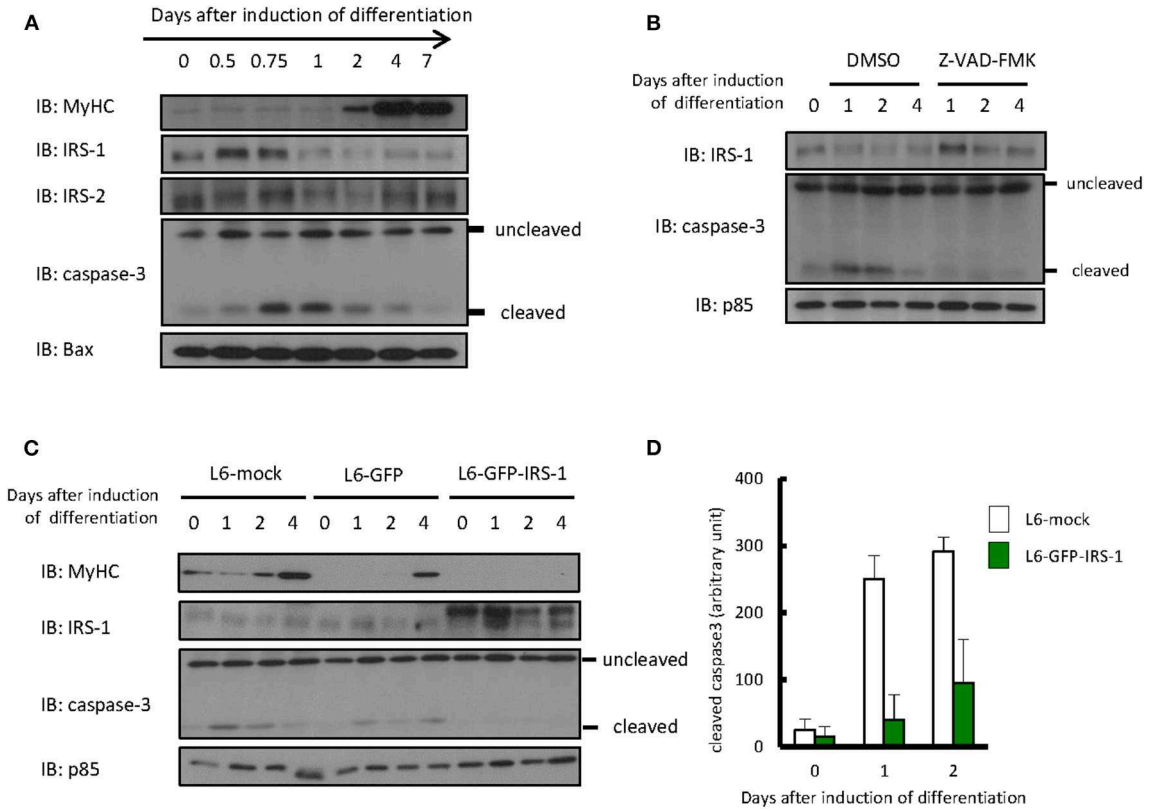

FIGURE 1 | Protein level of IRSs and cleaved caspase 3 during myogenic differentiation of L6 myoblasts. **(A)** Differentiation of L6 myoblasts was induced by changing media from DMEM with 10% FBS to DMEM with 2% FBS. At the indicated days after differentiation induction, cell lysates were prepared, and total cell lysates were produced for immunoblotting analysis using the indicated antibodies. **(B)** Differentiation was induced in the differentiation medium with or without 100 μM Z-VAD-FMK. Immunoblotting was conducted using the indicated antibodies at the indicated days after differentiation induction. **(C)** L6-mock, L6-GFP, and L6-GFP-IRS-1 were induced to differentiate into myotubes. Immunoblotting was conducted at the indicated days after differentiation induction. **(D)** At the indicated days after differentiation induction, cells were fixed by PFA and immunostained with anti-cleaved caspase 3 antibody. The number of cleaved caspase 3-positive cells was counted, and the data is shown as means ± SEM. These are representative data from experiments independently performed twice.

cells highly expressing IRS-1 are selectively excluded from the cell layer.

The Ability of Cells With Higher IRS-1 Levels to Attach to the Normal Cell Layer Was Impaired

Cells with high IRS-1 levels were eliminated upon culturing with normal cells. These data suggested that cell-cell contact plays important roles in this phenomenon. Thus, we examined the cell attachment ability of cells with high IRS-1 levels. Initially, L6-mock cells were seeded on the dish and cultured until confluent; then, L6-mycIRS-1-GFP or L6-GFP cells were seeded on the confluent L6-mock cell layers. As a control, L6-mycIRS-1-GFP cells or L6-GFP cells were seeded on the vacant dishes. The number of cells attached to the vacant dishes was comparable between L6-GFP and L6-mycIRS-1-GFP (**Figure 3A**). However, the number of L6-mycIRS-1-GFP attached on the L6-mock layer was significantly lower than that of L6-GFP (**Figure 3B**). The ratio of the GFP-positive cell number on the L6-mock cell layers to the number of GFP-positive cells on the vacant dishes was defined as CLAI. As a result, the cell attachment ability of L6-mycIRS-1-GFP to L6-mock was significantly lower than that of L6-GFP to L6-mock cells (**Figure 3C**).

Cells With Higher Levels of IRS-1 3YA Mutant Were Not Excluded Upon Culturing With Normal Cells

As shown in **Figure 2**, we showed that cells with higher levels of IRS-1 were selectively eliminated from the cell layer when cultured with normal cells. Recently, we reported that IGF-I receptor internalization was inhibited in L6 myoblasts with high IRS-1 levels, resulting in sustained activation of IGF signaling. In addition, we prepared L6 myoblasts stably expressing IRS-1 3YA mutant and GFP (L6-IRS-1 3YA), which did not sustain IGF signal activation since this mutant could not inhibit internalization of the IGF-I receptor (20). In L6-IRS-1 3YA, differentiation-induced Caspase 3 activation was not enhanced (**Figure S1**), and the growth rate was almost identical with normal cells (**Figure S2**). Next, L6-IRS-1 3YA was cultured with normal

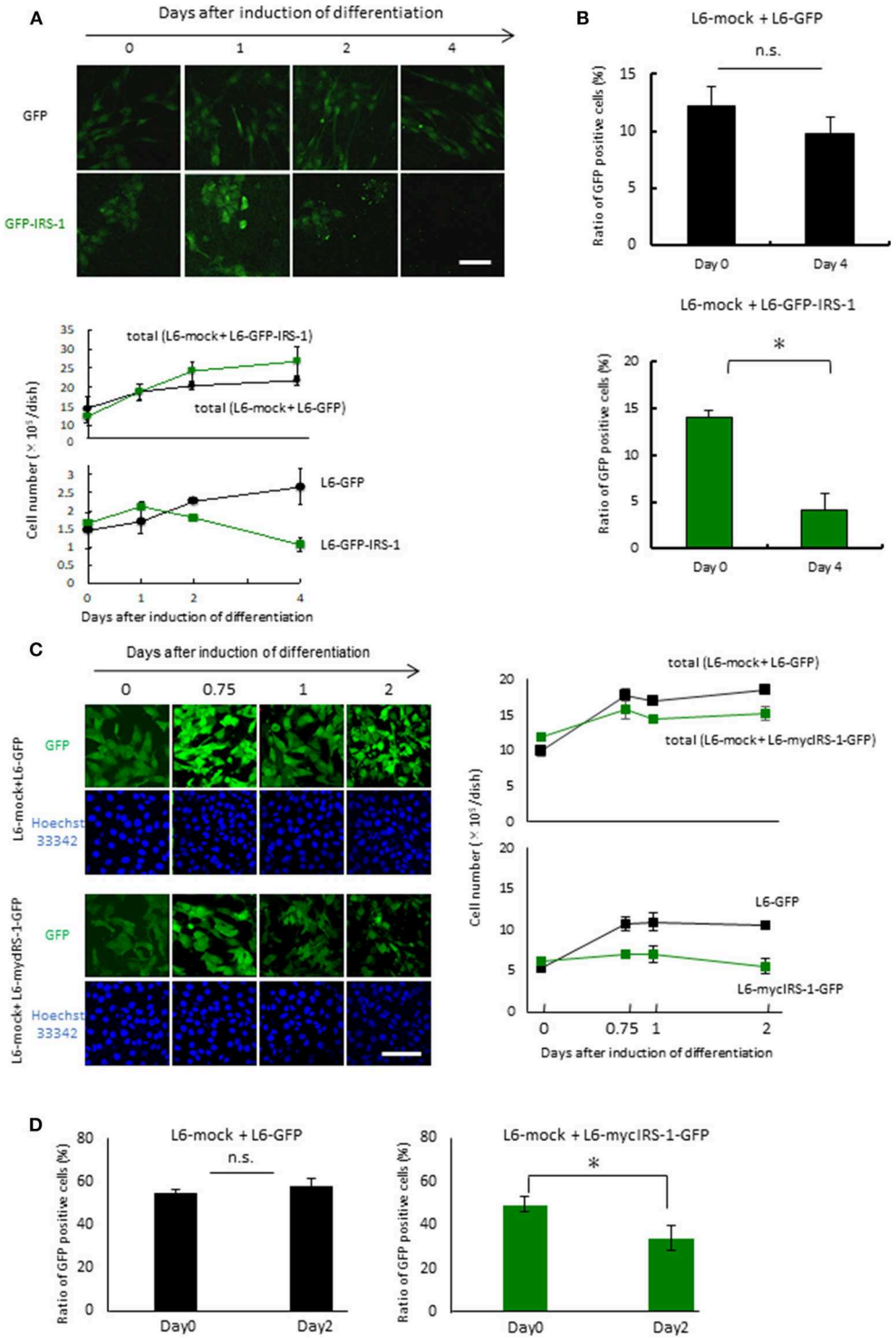

FIGURE 2 | Elimination of cells highly expressing IRS-1. **(A)** Mixtures of L6-GFP and L6-mock or L6-GFP-IRS-1 and L6-mock were inoculated into dishes at a 1:10 ratio, and differentiation was induced. At the indicated days after differentiation induction, cells were fixed by PFA, and the total nucleus numbers and the nucleus numbers of GFP-positive cells were counted (right graphs). Scale bar: 100 μm. **(B)** The percentage of GFP-positive cells was calculated at day 0 or 4 after differentiation induction, as shown in the graph. Data is shown as means ± SEM. *$p < 0.05$ vs. day 0. **(C)** Mixtures of L6-GFP and L6-mock or L6-mycIRS-1-GFP and L6-mock were inoculated into the dishes at ratios of 1:1, and differentiation was induced. At the indicated days after differentiation induction, cells were fixed by PFA, and the numbers of GFP-positive and GFP-negative cells were counted (lower graphs). Scale bar: 100 μm. **(D)** The percentage of GFP-positive cells was calculated at day 0 or 4 after differentiation induction, as shown in the graph. Data is shown as means ± SEM. *$p < 0.05$ vs. day 0. These are representative data from experiments independently performed at least three times.

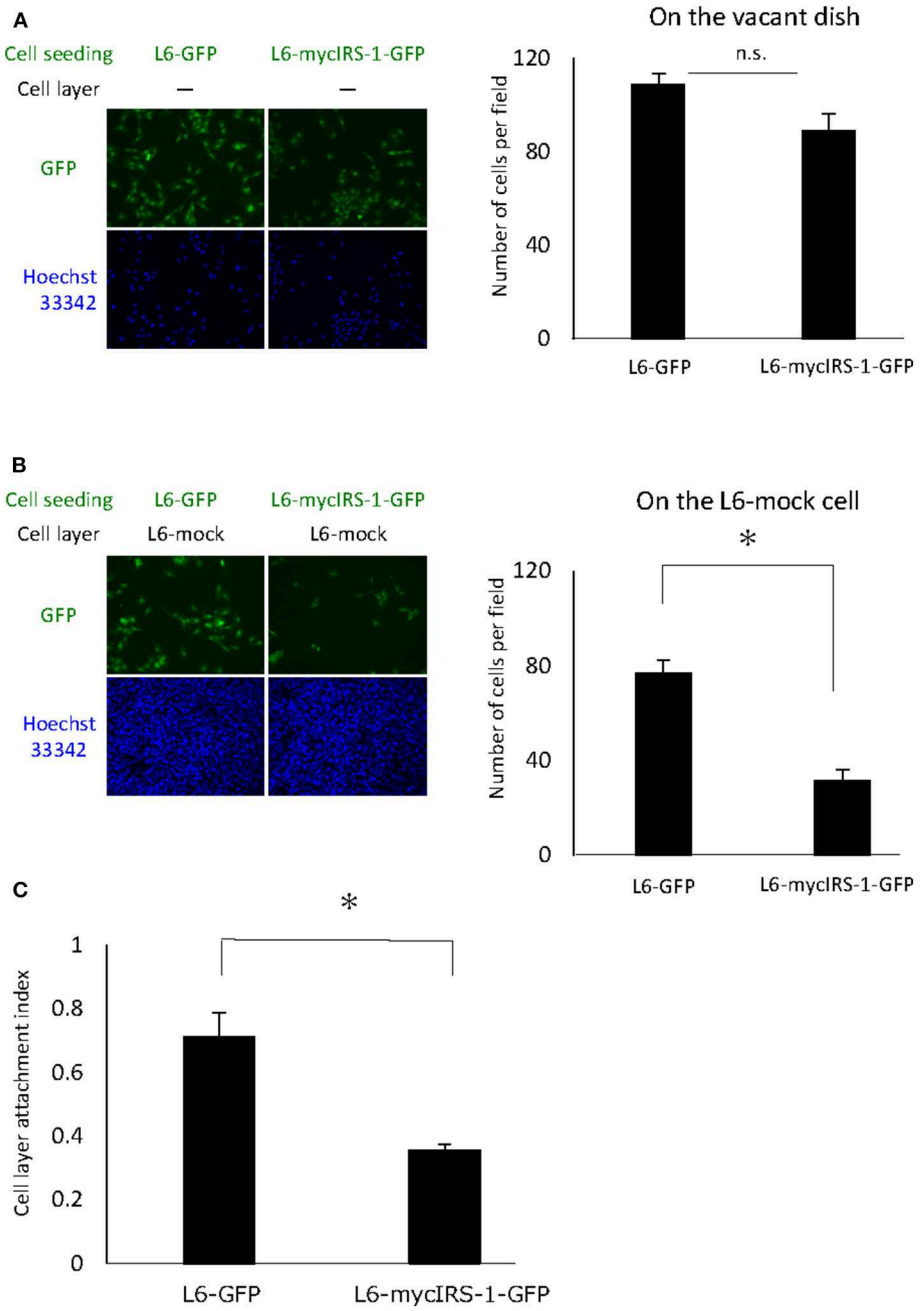

FIGURE 3 | Attachment ability of cells highly expressing IRS-1. **(A)** Five million L6-mock cells were seeded on the dish and cultured until confluent. Either L6-mycIRS-1-GFP or L6-GFP was then seeded on the L6-mock confluent cell layer or directly on a dish. After 1 day of incubation, the cells were fixed and the numbers of GFP-positive cells were counted. **(B)** CLAI was defined as shown. **(C)** CLAI was calculated, and data are shown as means ± SEM ($n = 3$), $^*p < 0.05$. These are representative data independently performed at least three times.

cells, and we tested cell elimination from the cell layer. As shown in **Figure 4A**, elimination from the cell layer was observed when L6-mycIRS-1-GFP was cultured with L6-mock, whereas this was not observed when L6-IRS-1 3YA was co-cultured with L6-mock (**Figures 4A,B**). Under this situation, the attachment ability of L6-IRS-1 3YA to L6-mock cells was unchanged (**Figures 4C,D**).

Finally, we performed the similar experiments under the growth medium. L6-GFP, L6-mycIRS-1-GFP or L6-IRS-1 3YA was cultured with L6-mock normal cells in the DMEM 10% FBS and counted the nucleus number at the indicated days. Growth rate until 2 days was very similar for all cell lines. L6-GFP and L6-IRS-1 3YA could increase cell number until 4 days whereas L6-mycIRS-1-GFP could not increase the cell number but decreased

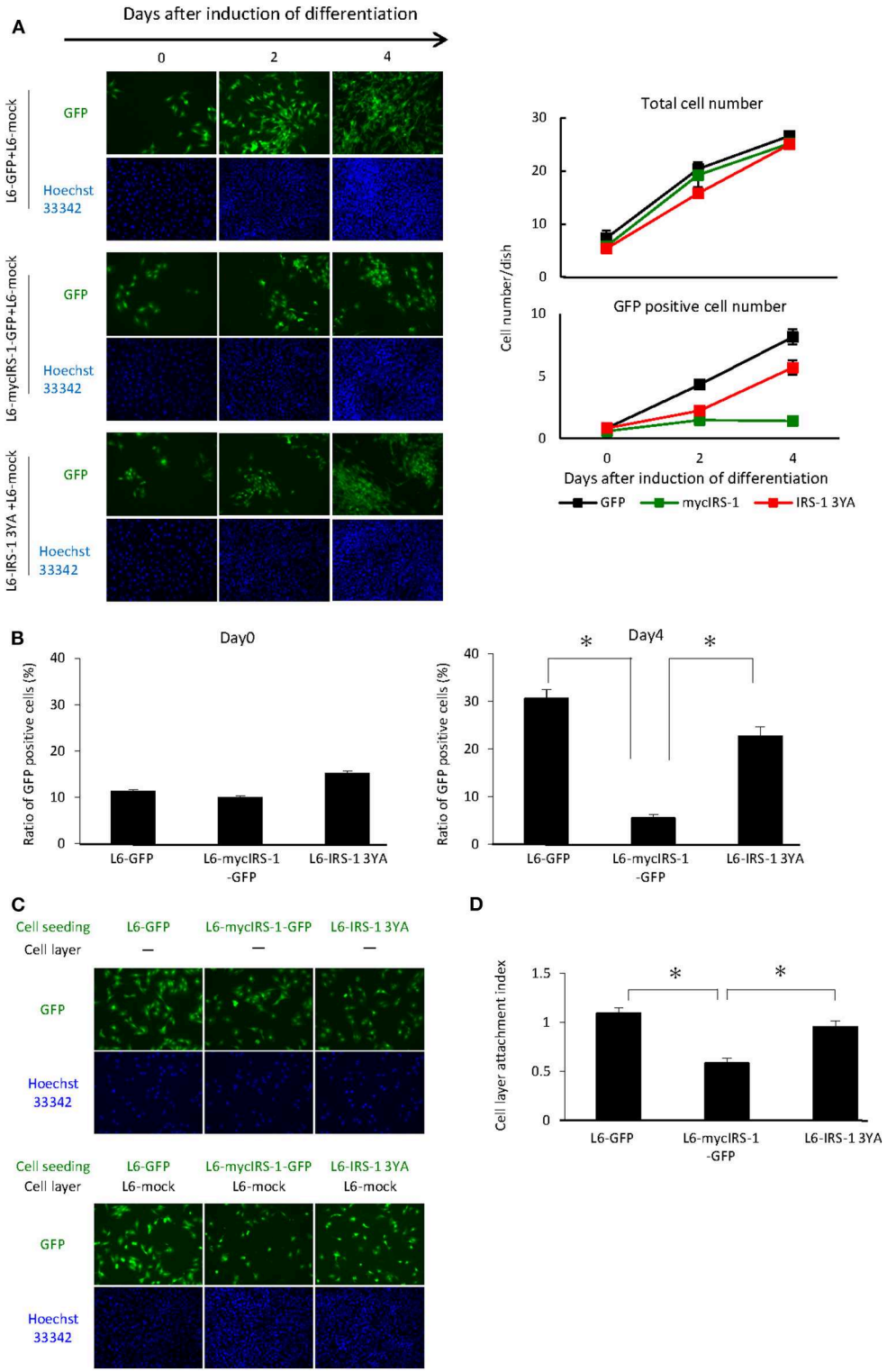

FIGURE 4 | Elimination of cells overexpressing IRS-1 3YA mutant. **(A)** L6-GFP, L6-mycIRS-1-GFP, or L6-IRS-1-3YA cells were co-cultured with L6-mock in differentiation medium at a 1:10 ratio. At the indicated days after differentiation induction, cells were fixed by PFA, and the total nucleus numbers and the nucleus numbers of GFP positive cells were counted (right graphs). **(B)** The percentage of GFP-positive cells was calculated at day 0 or 4 after differentiation induction, as shown in the graph. Data is shown as means ± SEM. *$p < 0.05$ vs. day 0. **(C)** Five million L6-mock cells were seeded on the dish and cultured until confluent. L6-mycIRS-1-GFP, L6-GFP, or IRS-1 3YA mutant cells were then seeded on the L6-mock confluent cell layer or on a dish. After 1 day of incubation, the cells were fixed and the numbers of GFP-positive cells were counted. **(D)** CLAI was calculated as previously indicated, and data are shown as means ± SEM ($n = 3$), *$p < 0.05$. These are representative data from experiments independently performed at least three times.

(**Figure S3**). This data indicated that cells overexpressing IRS-1 were eliminated from the cell layer also under growth condition.

DISCUSSION

In this study, we revealed that IRS-1 overexpressing cells are eliminated from the cell layer upon culturing with normal cells. A similar phenomenon, cell competition, was observed in *Drosophila* (21). Cell competition is a cell fitness sensing mechanism where a less fit cell is eliminated as a "loser" when surrounded by fitter cells, or "winners." In *Drosophila* imaginal discs, several mutants affecting cell proliferation including *Minute*, which have mutations in ribosomal genes, were reported to cause cell competition. Furthermore, cells with additional copies of *Myc* become "super-competitors" and can eliminate neighboring wild-type cells. Cells with mutant tumor suppressor genes *scribble* (*scrib*), *lethal giant larvae* (*lgl*), and *discs large* (*dlg*) lose cell polarity and are eliminated by the surrounding normal epithelial cells. In addition, recently, a Hippo signaling mutant caused cell competition in mammalian cells (22). Mammalian cells overexpressing active YAP1, which is a downstream protein of the Hippo signal, exhibit a cell-autonomous decrease in cell adhesion, and cell attachment to the culture dish influences the win-or-lose outcome of the competition with wild-type cells (23). In our L6 myogenic differentiation system, IRS-1-overexpressing cells are eliminated from the cell layer as loser cells. This is the first report that an IGF signaling protein was identified as a molecule that caused cell competition.

We have shown that a differential level of IRS-1 caused cell competition under a heterotypic population. However, we did not show that such cell competition could occur under physiological conditions. Recently, we reported the detailed molecular mechanism underlying the negative feedback loop of IGF signal transduction. In this paper, we present the precise mechanism of IGF-I-induced IRS-1 protein degradation. The downstream kinase mTORC1 phosphorylates the serine residue of IRS-1 at amino acid 422; this phosphorylation recruits ubiquitin ligase, resulting in IRS-1 degradation (19). Given our data, we envisage a possible scenario: since the IRS-1 protein level is dynamically changed in response to IGF stimulation and varies in each cell, cells with higher IRS-1 levels are eliminated under physiological conditions.

In this study, we have shown that attachment ability of IRS-1 overexpressing cells to L6-mock cells is lower than that of normal cells. These data suggest a possible mechanism of cell elimination of IRS-1 overexpressing cells. It is well-known that cells lose adhesion to dishes before cell division during proliferation. After cell division, divided cells invade into the cell layer again, but IRS-1 overexpressing cells cannot invade into the cell layer because of the low attachment ability to normal cells, resulting in selective elimination of IRS-1 overexpressing cells. Next question is "Do loser cells die?". Our data showed that IRS-1 overexpressing cells are specifically eliminated from the cell layer. And at this differentiation stage, apoptotic cells increased, suggesting that eliminated loser cells might die by apoptosis. In Drosophila, apoptotic signal is activated in loser cells, including the JNK pathway. But in our model, to identify the apoptotic pathway was required for the further evaluation.

In order for the loser cells to be recognized by the winner cells, some components that label cells as "losers" are required. However, since IRS-1 is an intracellular protein, it could not be the candidate component of direct recognition for cell competition. Recently, we showed that IRS-1 interacts with the medium chain of clathrin-coated adaptor protein (AP2), and this interaction inhibited AP2 function to facilitate ligand-induced IGF-I receptor internalization. Thus, IRS-1 overexpression inhibited IGF-I receptor internalization, resulting in sustained Akt/mTORC1 activation (20). In this study, overexpression of IRS-1 mutant 3YA, which is an AP2-binding-deficient mutant, did not delay AP2-mediated IGF-IR endocytosis after ligand stimulation and did not cause cell competition. These data suggested that the accumulation of active IGF-I receptors on the plasma membrane labels cells as the loser cells. In addition, we showed that cell attachment to normal cells is inhibited in cells highly expressing IRS-1. It was reported that integrin and cadherin were bound to IRS-1 and the IGF-I receptor (24–26), and these molecules play roles in cell-cell or cell-extracellular matrix binding (27, 28). Moreover, Canonici et al. explained that IGF-I modulates association between IGF-I receptor, αv integrin and E-cadherin (29). These reports suggested that integrin and cadherin could be candidate components for labeling cells as the "losers."

What is the relationship between cell competition and myogenic differentiation? Some reports demonstrated that apoptosis induced by the induction of myogenic differentiation was required for myoblast cell fusion (13). We expect that apoptosis induced by cell competition is also required for myoblast differentiation. Actually, we have reported that IRS-1 overexpression inhibited myogenic differentiation, and also demonstrated that continuous inhibition of Foxo1 due to sustained Akt activation caused defect of myogenesis possibly through repression of MyHC expression in IRS-1 overexpressing cells at the late stage of the differentiation process (12). These strongly suggested that defect of myogenesis in IRS-1 overexpressing cells was not only caused by disturbance of cell competition which happened in the beginning of differentiation. Cell competition might be a possible mechanism to prevent the subpopulation of myoblasts with high-level of IRS-1 from differentiating into myotubes. However, further analysis is required to evaluate involvement of cell competition in myogenic differentiation.

Why was cell competition induced by differential IRS-1 protein levels in myoblast cells? Skeletal muscle differentiation entails the coordination of muscle-specific gene expression and terminal withdrawal from the cell cycle, inducing permanent G1 phase. The execution of this pathway is required for the formation of multinucleated myotubes (30–33). These findings suggested that the cell cycle of myoblasts that were fusing to myotubes was adjusted to the G1 phase. It is well-known that in a variety of muscle types, IGF-I regulates proliferation through its effects on the cell cycle (34, 35). Furthermore, it was recently revealed that apoptotic myoblasts enhanced fusion (13). Based on these papers, we propose the hypothesis that

cell competition monitored by the IRS-1 level induces apoptotic cells, in which the IGF signal and cell cycle phase differ from neighboring cells. Then, in the surviving cells, the IGF signal and cell cycle phase are easily synchronized, and these synchronized cells fuse to myotubes. Thus, it is possible that cell competition during myogenesis plays important roles for the functionally synchronized cells to fuse to myotubes.

We showed that myoblasts expressing high IRS-1 levels were eliminated upon culturing with normal cells. Furthermore, the sustained activity of the IGF-I receptor on the plasma membrane might be the signal for the loser cells. This mechanism can explain why cell proliferation and cell apoptosis can be induced at the same period during myogenic differentiation. We also found that the decrease in IRS-1 was also induced in adipogenesis (unpublished data). These results suggested that the decreases in IRS-1 and IGF signaling by IGF-I receptor downregulation could be crucial for cell differentiation.

L6 myoblasts that highly expressed IRS-1 protein were eliminated from the cell layer upon culturing with normal cells due to sustained activation of the IGF-I receptor on the plasma membrane. It is possible that cell competition induced by the differential level of IRS-1 is required for myogenic differentiation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FH contributed to the conception and design of the study. RO and AU performed the experiments. S-IT wrote the manuscript. All authors discussed the results and approved the manuscript.

FUNDING

This work was supported in part by the Japan Society for the Promotion of Science (JSPS) Fellows and for Young Scientists (B) #15K18766 from JSPS to YY; Grant-in-Aid for Scientific Research (#24380152, #15H04583) to FH; Grant-in-Aid for Scientific Research (S) #25221204, (A) #18H03972, and core-to-core program A. A. Advanced Research Network to S-IT; and R&D matching funds on the field for Knowledge Integration and Innovation from Bio-oriented Technology Research Advancement Institution, NARO, to S-IT.

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.com) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00096/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 03 March 2020
doi: 10.3389/fendo.2020.00100

Insulin-Like Growth Factor Binding Protein-5 in Physiology and Disease

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Insulin-like growth factor (IGF) signaling is regulated by a conserved family of IGF binding proteins (IGFBPs) in vertebrates. Among the six distinct types of IGFBPs, IGFBP-5 is the most highly conserved across species and has the broadest range of biological activities. IGFBP-5 is expressed in diverse cell types, and its expression level is regulated by a variety of signaling pathways in different contexts. IGFBP-5 can exert a range of biological actions including prolonging the half-life of IGFs in the circulation, inhibition of IGF signaling by competing with the IGF-1 receptor for ligand binding, concentrating IGFs in certain cells and tissues, and potentiation of IGF signaling by delivery of IGFs to the IGF-1 receptor. IGFBP-5 also has IGF-independent activities and is even detected in the nucleus. Its broad biological activities make IGFBP-5 an excellent representative for understanding IGFBP functions. Despite its evolutionary conservation and numerous biological activities, knockout of IGFBP-5 in mice produced only a negligible phenotype. Recent research has begun to explain this paradox by demonstrating cell type-specific and physiological/pathological context-dependent roles for IGFBP-5. In this review, we survey and discuss what is currently known about IGFBP-5 in normal physiology and human disease. Based on recent *in vivo* genetic evidence, we suggest that IGFBP-5 is a multifunctional protein with the ability to act as a molecular switch to conditionally regulate IGF signaling.

Keywords: IGF signaling, AKT, mTOR, PAPP-A, STC, IGF-dependent, IGF-independent action

OPEN ACCESS

Edited by:
Claire Perks,
University of Bristol, United Kingdom

Reviewed by:
Robert C. Baxter,
University of Sydney, Australia
Briony Forbes,
Flinders University, Australia

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Specialty section:
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *06 January 2020*
Accepted: *17 February 2020*
Published: *03 March 2020*

Citation:
Duan C and Allard JB (2020) Insulin-Like Growth Factor Binding Protein-5 in Physiology and Disease. Front. Endocrinol. 11:100. doi: 10.3389/fendo.2020.00100

INTRODUCTION

The insulin-like growth factors (IGFs), including IGF-1 and IGF-2, are peptides that act throughout the vertebrate body via endocrine, paracrine, and autocrine signaling. IGFs bind to the IGF-1 receptor (IGF1R), a receptor tyrosine kinase that structurally resembles the insulin receptor (1). IGFs have very low affinity for the insulin receptor. The IGF signaling pathway regulates cell survival, differentiation, migration, and proliferation at the tissue level, and somatic growth, developmental progression, and aging at the organismal level (2–7).

A family of IGF-binding proteins (IGFBPs), regulates IGF bioavailability by binding to IGF ligands with equal or higher affinity than the IGF1R (8). Almost all of the IGFs in the extracellular environment, both in tissues and in the circulation, are found in complexes with IGFBPs (9, 10). There are six distinct types of IGFBPs in vertebrates, labeled IGFBP-1 through IGFBP-6. IGFBPs are found in all vertebrates studied to date, though not all species possess genes of all six IGFBP types, and some have multiple gene paralogs of some or all of the types (11). We have recently discussed the question of why the IGFBP family comprises such a large number of genes with substantial functional redundancy (11).

Here we review the current understanding of the structure, expression, regulation, and biological actions of IGFBP-5, which is the most highly conserved IGFBP family member. Like other IGFBP family members, IGFBP-5 binds to IGFs and can act to inhibit the interaction of the IGFs with the IGF1R and thereby reduce IGF signaling activity (11). While all IGFBPs have both shared and unique biological capabilities, spatiotemporal expression patterns, post-translational regulatory mechanisms, protein-protein interaction partners, etc., IGFBP-5 has one of the most diverse sets of biological actions of any IGFBP. This rich repertoire of activities makes IGFBP-5 an ideal representative of the IGFBP family by which to illustrate the range of mechanisms by which IGFBPs can modulate and fine-tune IGF signaling, and also carry out incidental functions that are independent of IGF binding. IGFBP-5 has been investigated for decades and discussed in a vast literature. We discuss evidence from a variety of vertebrate species, and in order to avoid confusion resulting from different gene/protein nomenclature systems, we will use the name "IGFBP-5" in all cases and explicitly indicate the species where necessary.

STRUCTURE AND FUNCTIONAL MOTIFS

IGFBP-5 was first identified and purified from human bone extracts and conditioned media collected from cultured human osteosarcoma cells (12, 13). It was subsequently cloned and characterized in a variety of vertebrate species (14–16). IGFBP-5 is found in all vertebrates studied to date, and its orthologs generally share the highest levels of amino acid sequence identity of any of the IGFBP types. Human IGFBP-5 contains 272 amino acids and most mammalian homologs of IGFBP-5 have either 272 or 271 amino acids. Human and zebrafish IGFBP-5 have an overall 55% sequence identity. Like all IGFBPs, mature human IGFBP-5 (252 amino acids) has a primary structure consisting of 3 domains, a highly conserved N-terminal domain, an unstructured linker (L-) domain that acts as a hinge, and a C-terminal structured domain that contains a thyroglobulin type-I repeat (11, 17) (**Figure 1**). The N- and C-terminal domains (N- and C- domains) are structurally stabilized by intradomain disulfide bonds between cysteine residues that are conserved across species, with 12 residues in the N- domain and 6 in the C domain (**Figure 1**). The L-domain is the least conserved region of the protein (17, 18).

Both the N- and C-domains in IGFBP-5 participate in IGF binding, but a fragment of the N-domain was found to bind IGF with substantial affinity, suggesting that it contains a sizable part of the interaction surface (19). The C-domain contains a highly conserved KR-rich sequence that overlaps with areas that contribute to IGF binding (20) (**Figure 1**). Binding of this region to heparin reduces the affinity for IGF by 17-fold, promoting release of bound IGF (21, 22). This region is also important for nuclear localization of IGFBP-5 (16, 23, 24) and ALS association (17, 25) (**Figure 1**). The C-domain also contains sites for binding various components of the extracellular matrix (ECM) (22). IGFBP-5 has been found to be localized within the ECM in tissues and has been shown to bind directly to a number of ECM proteins including types 3 and 4 collagen, Laminin, Fibronectin, Plasminogen Activator Inhibitor 1, Thrombospondin, and Osteopontin (26–28). The functional significance of ECM binding is discussed further below. The L-domain contains a number of proteolytic cleavage sites, phosphorylation, and O-glycosylation sites (17, 18, 29–31).

EXPRESSION AND REGULATION

IGFBP-5 is expressed in a variety of different tissues throughout the body including lung, bone, muscle, testis, ovary, kidney, etc., with variations in different developmental stages and between species (18). IGFBP-5 expression is regulated by hormones in certain tissues and cell types, and is expressed constitutively

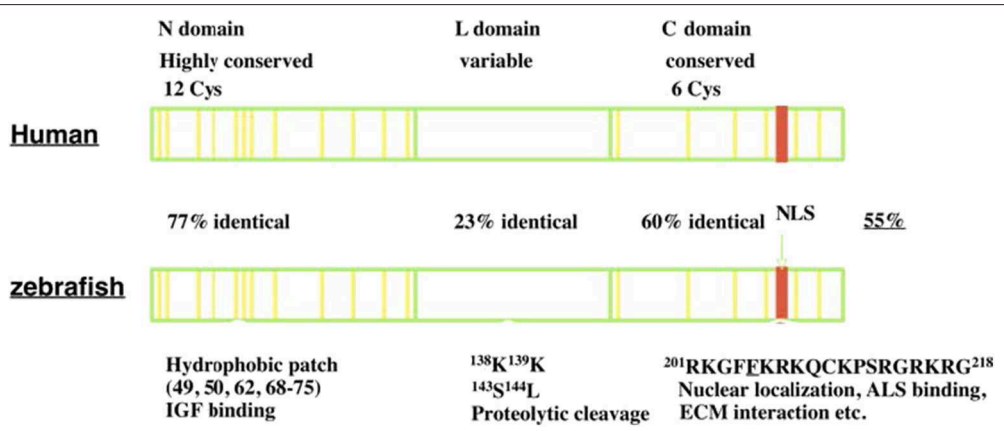

FIGURE 1 | The structure of IGFBP-5. IGFBP-5 consists of 3 structurally domains: a highly conserved globular N-terminal domain, a central non-conserved linker domain, and a conserved C-terminal globular domain. The N-domain contains 12 conserved cysteine residues and a hydrophobic patch important for IGF binding. The L-domain contains several proteolytic cleavage sites. The C-domain contains six conserved cysteine residues, a RK-rich sequence (red) important for IGF binding, ALS binding, nuclear localization, and a thyroglobulin-like fold and other sites of interaction with ECM components.

in others. For instance, in mammary gland cells, IGFBP-5 expression is inhibited by prolactin (32). In osteoblast-like cells, parathyroid hormone upregulated IGFBP-5 expression (33). IGFBP-5 expression can also be upregulated by IGF signaling in vascular smooth muscle cells and other cell types (34, 35). In mouse mammary gland, IGFBP-5 expression is suppressed by the hormone prolactin and STAT-3 (32, 36, 37). IGFBP-5 mRNA has also been found to be regulated by several miRNAs (37–40).

Post-translational mechanisms also regulate IGFBP-5 in important ways (18). IGFBP-5 was shown to be phosphorylated on several serine residues *in vivo,* which reduced its binding affinity for heparin but not for IGFs (41). In the extracellular environment, a number of specific proteases cleave IGFBP-5. In some cases, proteolysis of IGFBP-5 is inhibited by IGF binding (42). The zinc-dependent metalloproteinases pregnancy-associated plasma protein-a (PAPP-A) and PAPP-A2 have been shown to cleave IGFBP-5 at a single site in the L-domain (31, 43, 44). Unlike IGFBP-4, which is only susceptible to cleavage by PAPP-A when it is bound to IGF, IGFBP-5 is cleaved by both proteases regardless of IGF binding (31, 43). PAPP-A2 knockout mice had 2-fold higher levels of IGFBP-5. Interestingly, these mice also exhibited a 15-fold reduction in IGFBP-3 levels and a 60% increase in total IGF levels (45). The deletion of PAPP-A2 in osteoblast cells in mice led to a significant reduction in growth as measured by both body mass and tail length (46). The proteolytic regulation of IGFBP-5 by PAPP-A and PAPP-A2 is conserved in zebrafish (47). In addition to PAPP-A and PAPP-A2, a number of other proteases have been reported to degrade IGFBP-5. These include thrombin, elastase, cathepsin G, C1s, ADAM 9, ADAM 12s, MMP-1, and MMP-2 etc. (48–53).

Interactions with extracellular matrix (ECM) and cell surface proteins are also important for IGFBP-5 activity. A number of studies have demonstrated a link between IGFBP-5 ECM binding and its enhanced potentiation of IGF signaling. IGFBP-5 associated with the cell culture substratum of fibroblasts was found to potentiate the cellular growth promoting effects of IGF signaling (26). Binding to the ECM component vitronectin enhanced IGFBP-5's potentiation of IGF signaling in smooth muscle cells, and a mutant form of IGFBP-5 that did not bind to vitronectin did not produce this effect (54). IGFBP-5 mutants with reduced ECM binding ability had a reduced ability to potentiate IGF signaling *in vitro* (30, 55). Some ECM components also influence the biosynthesis of IGFBP-5. The ECM component fibronectin in the culture substrate was found to upregulate expression and secretion of IGFBP-5 in porcine smooth muscle cells (56).

Early *in vitro* studies showed that its binding to heparin-like glycosaminoglycans protected IGFBP-5 from proteolytic degradation in media conditioned by human dermal fibroblasts (57). This mechanism may allow IGF/IGFBP-5 complexes bound to proteoglycans in the ECM to avoid proteolysis for an extended period. It was suggested that IGFBP-5 may serve as a reservoir of IGF in tissues for later release when needed (30, 58–60). This may be important in bone tissue, where the IGF-IGFBP-5 complex is found in large quantities, binding to hydroxyapatite (58–60). A recent study found that IGF-1 released from the bone matrix promotes the differentiation of mesenchymal stem cells into osteoblasts, aiding bone formation during bone remodeling (61).

ENDOCRINE ROLE OF IGFBP-5

In adult human blood, IGFs are found at mean concentrations that are around 1,000-fold higher than insulin (9). Therefore, despite the low cross-reactivity of IGFs with the insulin receptor, if all circulating IGFs were free to interact, the hypoglycemic effects would overwhelm the effects of insulin itself. In addition, free IGF has a half-life in circulation of around 10 min (4, 62). Around 1% or less of circulating IGFs are free, and the remaining >99% are complexed with one of the IGFBPs (9). Binary complexes of IGF and IGFBP extend the half-life of the IGF to roughly 30 min, but they also facilitate the departure the IGF to be delivered to tissues (4, 62). Like IGFBP-3, which is the dominant IGFBP in the circulation, IGFBP-5 can bind to IGF alone or in a ternary complex with IGF and an 85 kDa glycoprotein called Acid Labile Subunit (ALS) (25). IGF within the ternary complex has a greatly prolonged half-life, and the complex is too large (around 150 kDa) to exit from the circulation, and thus it is able to maintain a circulating reservoir of IGF (63) (**Figure 2A**). About 75–80% of circulating IGF is found in a ternary complex with IGFBP-3 or−5 (9). When both IGFBP-3 and IGFBP-5 were knocked out in mice (64), or when ALS itself was deleted (65), the ternary complex was absent, and serum IGF levels were greatly reduced. However, there was only a modest reduction in growth, due to compensatory mechanisms by other IGFBPs.

Global overexpression of IGFBP-5 in mice, which resulted in a ∼4-fold increase in circulating IGFBP-5, caused a severe body growth reduction both prenatally and postnatally, significant neonatal mortality, reduction of fertility in females, and a 30% reduction in skeletal muscle weight (66). This phenotype was consistent with the notion that IGFBP-5 inhibits IGF signaling by sequestering IGF away from the IGF1R (4, 10). However, the relevance of this overexpression phenotype to the physiological role(s) of endogenous IGFBP-5 is unclear. Knockout of IGFBP-5 in mice did not result in altered body growth compared with wildtype, and it was assumed that other IGFBPs compensate for the lack of IGFBP-5 (67). This is supported by that fact that mice lacking IGFBP-3,−4, and−5 had somewhat reduced growth, changes in metabolism, a significant reduction in circulating and bioactive IGF-1 levels, which may explain the reduced body growth (64). Another possible explanation is that altered physiological and/or pathological states resulting from global dysregulation of IGF signaling led to growth inhibition.

ROLES OF LOCAL IGFBP-5 IN REGULATING IGF ACTIONS

Locally expressed IGFBP-5 can inhibit or enhance IGF biological activity by modulating their interaction with the IGF-I receptor (**Figures 2B,C**). IGFBP-5 is the most abundant IGFBP in bone tissues (68) and there is a host of *in vitro* findings in the literature regarding IGFBP-5 actions in osteosarcoma cells.

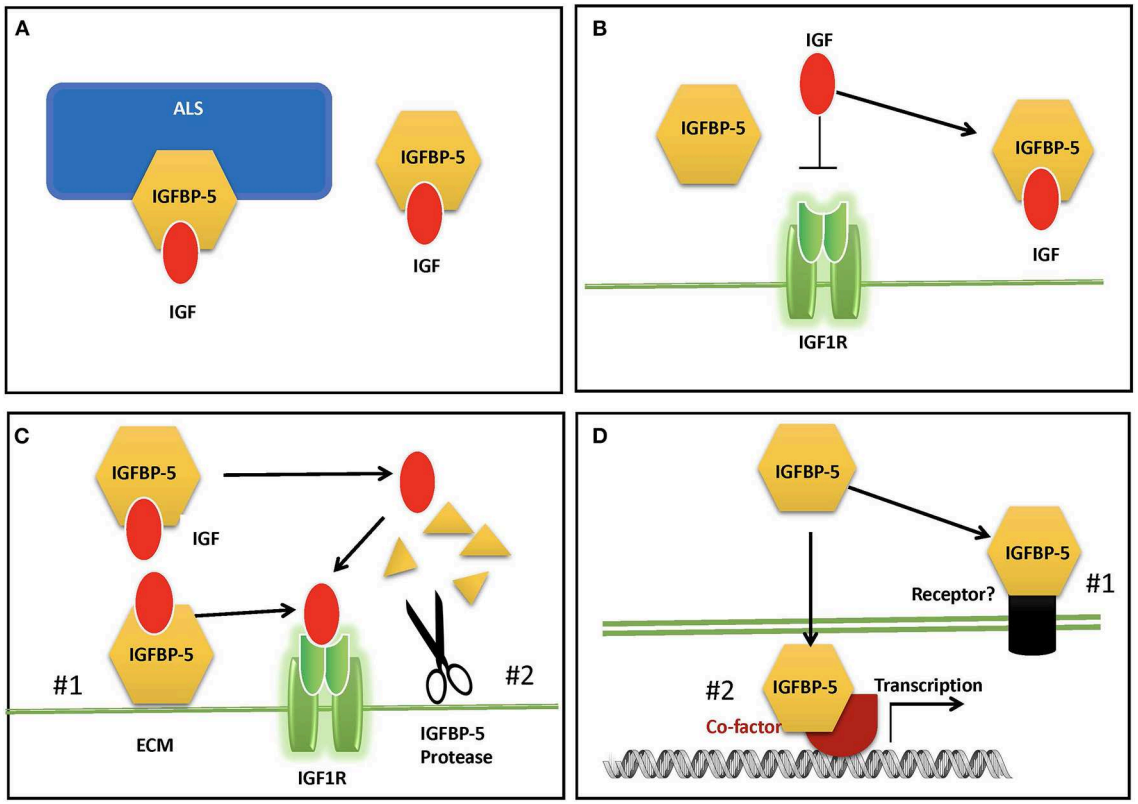

FIGURE 2 | Proposed modes of IGFBP-5 actions. **(A)** IGFBP-5 modulates circulating IGFs by forming a binary complex with IGF or a ternary complex with IGF and acid labile subunit (ALS) in the blood. **(B)** IGFBP-5 inhibits IGF signaling in target cells by sequestrating IGF away from the IGF1R. **(C)** IGFBP-5 potentiates IGF signaling in target cells by #1) releasing of bound IGF to the IGF-1R upon interaction with ECM and cell surface molecules or #2) protease-mediated IGFBP-5 proteolysis. **(D)** IGF-independent action of IGFBP-5 via #1) its putative membrane receptor(s) or #2) interacting with co-factors in the nucleus.

When added in combination with IGF-I to cultured human osteosarcoma cells, IGFBP-5 was found to inhibit IGF-I-induced cell growth [(69); 29]. Likewise, stable overexpression of IGFBP-5 was found to inhibit mouse osteosarcoma cell proliferation (70). In mesenchymal stem cell cultures, exogenously added IGFBP-5 and endogenously overexpressed IGFBP-5 inhibited osteoblast differentiation, while an IGF-binding deficient IGFBP-5 mutant did not have this effect (71). When IGFBP-5 was overexpressed in transgenic mice under the control of a bone specific osteocalcin promotor, osteoblast function was impaired, leading to reduced trabecular bone volume and reduced mineral densities (72). On the other hand, IGFBP-5 was also found to potentiate IGF-I-induced DNA synthesis and differentiation in bone cells (12, 13, 73, 74). The potentiating effects of IGFBP-5 were attributed to its ability to bind to the bone extracellular matrix (ECM) since IGFBP-5 has a high affinity for hydroxyapatite (73, 75). Given that IGFBP-5 is already abundantly expressed in bone cells, interpretations of data from the addition of exogenous IGFBP-5 or overexpression of IGFBP-5 are not always straightforward. Indeed, IGFBP-5 knockout mice had minimal changes in bone (64). Another complication is the presence of one or more IGFBP-5 protease(s) secreted by these cells and the fact that some IGFBP-5 fragments can exert IGF-independent actions in bone cells [(44, 76, 77), see below]. Moreover, different IGFBP-5 fragments might have different activities in osteosarcoma cells: while the N-terminal domain fragment inhibited cell proliferation and induced apoptosis, its C-terminal domain inhibited cell migration and metastases (78).

Yin et al. (79) investigated the role of endogenous IGFBP-5 using a siRNA based gene knockdown approach. They found that knockdown of IGFBP-5 increased osteosarcoma cell apoptosis. To further elucidate the mechanism underlying this action of IGFBP-5, we recently generated an expression plasmid encoding a siRNA resistant form of human IGFBP-5 (BP-5::GFP) (**Figure 3A**). The introduction of the siRNA-resistant IGFBP-5 into IGFBP-5 knocked down cells rescued cells from apoptosis (**Figures 3B,D**), suggesting that IGFBP-5 is both required and sufficient for maintaining osteosarcoma cell survival. IGFBP-5 is not only secreted but also localized in the nuclei and the IGFBP-5 has nuclear activity (see below). To determine the mechanism underlying IGFBP-5 actions, a ligand-binding deficient (LBD) and a nuclear localization deficient (NLS) form of IGFBP-5 (i.e., LBD::GFP and NLS::GFP) were engineered in the BP-5::GFP plasmid background (**Figure 3A**). The NLS mutant bound normally to IGF-1 but had greatly reduced

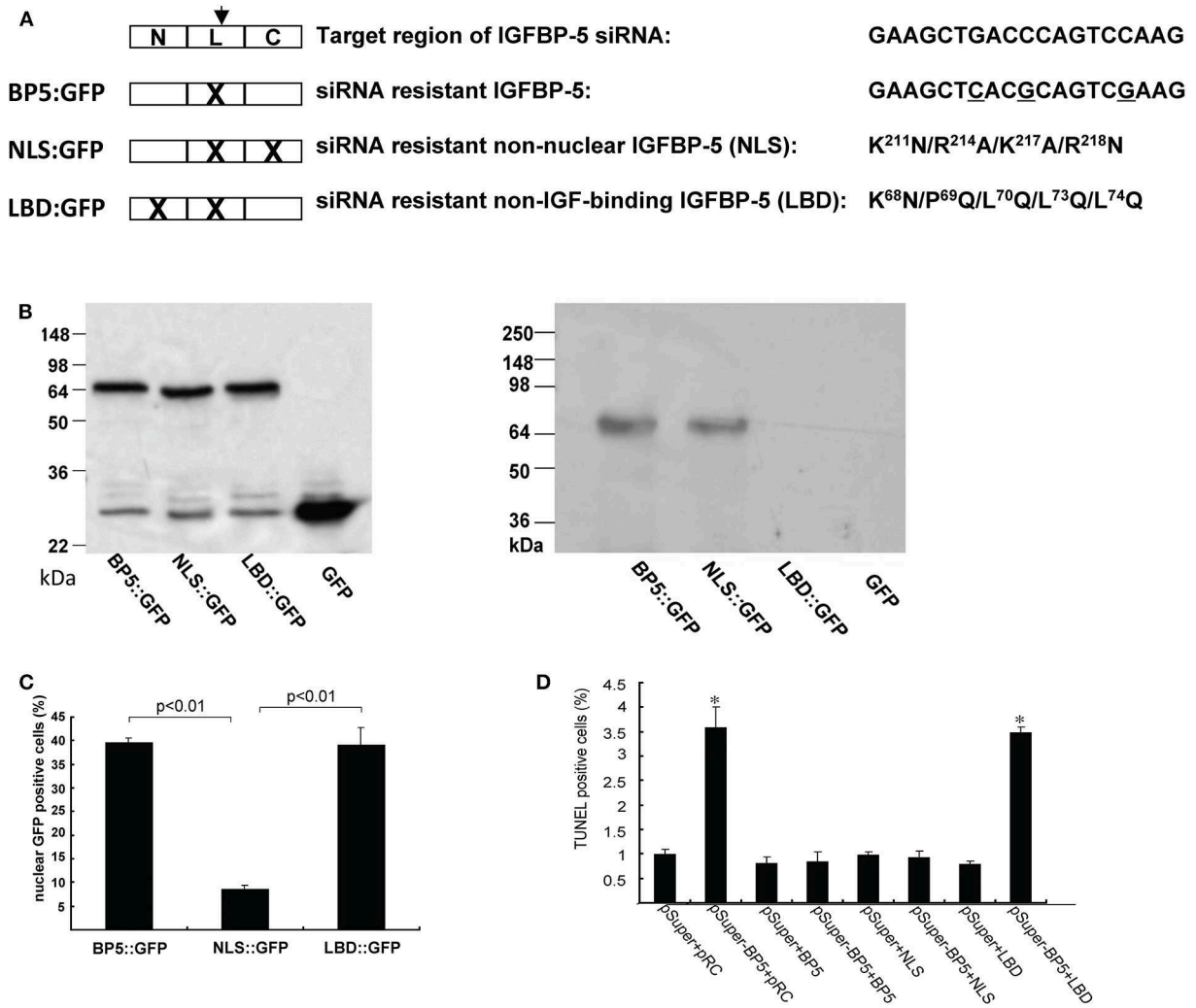

FIGURE 3 | IGFBP-5 regulates osteosarcoma cell survival by binding to IGFs. **(A)** Schematic diagram showing the structure of IGFBP-5 and three siRNA resistant IGFBP-5 expression constructs. Three point mutations were introduced into the target region of the siRNA to make the IGFBP-5 resistant to the RNA interference (BP5::GFP). Since the mutations are on the third position of the codons, the amino acid sequence is unchanged. Nuclear localization mutations (NLS) and IGF ligand binding deficient mutations (LBD) were further introduced into the siRNA resistant BP5::GFP plasmid, resulting in the NLS::GFP and LBD::GFP construct. **(B)** Left panel: Western immunoblot showing the expression levels of the three siRNA resistant IGFBP-5::GFP proteins in transfected U2 osteosarcoma cells. Right panel: Western ligand blot using DIG labeled IGF-I showing the ligand binding capability of BP5::GFP, NLS::GFP and LBD::GFP. Note the lack of IGF binding of LBD::GFP. pRC is the empty GFP vector. **(C)** Percentage of transfected cells with nuclear GFP signal. **(D)** The three siRNA resistant constructs were co-transfected into human U2 osteosarcoma cells with pSuper (the empty siRNA vector) or pSuper-BP5 (IGFBP-5 siRNA plasmid). The percentages of TUNEL positive cells were quantified. *$p < 0.05$ compared with the pSuper control group.

nuclear localization. The LDB mutant failed to interact with IGF-I, but showed similar nuclear localization (**Figures 3B,C**). The introduction of NLS::GFP but not LBD::GFP into IGFBP-5 knocked down human osteosarcoma cells rescued them from apoptosis (**Figure 3D**). These results suggest that endogenous IGFBP-5 regulates osteosarcoma cell survival by binding to IGFs and enhancing IGF action.

Involution of the mammary gland is the process by which a burst of apoptosis of mammary epithelial cells accompanied by ECM remodeling returns the gland to its condition before pregnancy (80). IGFBP-5 is upregulated in mammary epithelial cells during involution, where it may inhibit IGF signaling (32, 81, 82). This reduction of IGF signaling removes a key survival signal and thereby promotes mammary cell apoptosis (81). Indeed, IGFBP-5 knockout mice exhibited delayed mammary gland involution, as well as an enhancement in alveolar bud formation when ovariectomized mice were regularly injected with ovarian hormones to strongly promote mammary development (67). Transgenic overexpression of IGFs in mammary tissue led to a delay in involution (83, 84). Overexpression of IGFBP-5 in mammary gland resulted in a 50% reduction in mammary cell number and milk production, along

with a reduction in the activation of downstream IGF signaling, an increase in expression of the proapoptotic caspase-3 and a decrease in expression of antiapoptotic components (85). These findings suggest that IGF-1 promotes alveolar bud formation in normal pubertal mammary gland development and inhibits mammary cell apoptosis, and that IGFBP-5 inhibits these IGF actions (67). The upregulation of IGFBP-5 has been found to promote apoptosis in other tissues as well, including neurons and cardiomyocytes (86, 87).

Genetic studies in zebrafish have shown that IGFBP-5 potentiates IGF signaling in epithelial cells *in vivo* (88). One of the two zebrafish IGFBP-5 paralogous genes, IGFBP-5a is specifically expressed in a population of epithelial cells, known as Ca^{2+} transporting ionocytes or NaR cells, whose role is to take up Ca^{2+} from the aquatic environment to maintain body Ca^{2+} homeostasis (89–91). When environmental [Ca^{2+}] becomes scarce, these normally non-dividing and quiescent ionocytes reenter the cell cycle and begin to proliferate, producing a much larger capacity for Ca^{2+} uptake and allowing the embryos/larvae to survive under these stressful conditions (90, 92). This proliferative response is mediated by IGF signaling which is activated exclusively in these cells in response to low Ca^{2+} stress (90, 92). Genetic deletion of IGFBP-5a prevented the activation of IGF signaling in ionocytes under low [Ca^{2+}] stress (88). This prevented the adaptive proliferation of ionocytes, and the IGFBP-5 null embryos were therefore unable to survive under low [Ca^{2+}] stress (88). Reintroduction of wild-type zebrafish IGFBP5a in the mutant cells restores their adaptive proliferation. However, a ligand binding deficient IGFBP5a mutant had no such effect, suggesting that locally expressed IGFBP-5a regulates epithelial cell proliferation by binding to the IGF ligand and promoting IGF signaling under low [Ca^{2+}] stress. This action appears to be conserved in human cells because knockdown of IGFBP-5 expression in human colon carcinoma cells reduced their proliferative response to IGF-2 stimulation (88). *In vivo*, expression of human IGFBP-5 in mutant zebrafish increased ionocyte proliferation, whereas two cancer-associated human IGFBP-5 mutations with impaired IGF binding ability (93) had no effect (88). This type of local regulation of IGF signaling by IGFBP-5 under certain stressful and/or pathophysiological states may be a common mechanism. It was reported that castration of male mice induces local IGFBP-5 expression in prostate tissue and the elevated IGFBP5 increases IGF action and promotes prostate cancer progression (94, 95). Likewise, an increase in local IGFBP-5 expression has been shown in resection-induced adaptive colon growth (96).

Another example of the IGF potentiating effects of IGFBP-5 is seen in muscle development, where IGF-2 has been found to promote proliferation of myoblast cells as well as their differentiation into mature muscle cells (97). Myoblasts secrete IGF-2 during differentiation, which acts in an autocrine fashion (98). Upregulation of IGFBP-5 preceded upregulation of IGF-2 in these cells and knockdown of IGFBP-5 blocked myogenic differentiation, suggesting that IGFBP-5 was necessary to guide the activity of IGF signaling toward differentiation (99). This action of IGFBP-5 required its ability to bind to IGF-2 because an IGF-binding deficient form of IGFBP-5 had no such effect (99). IGFBP-5 was found incorporated into the ECM in cultures of fetal fibroblasts, and ECM binding facilitated its potentiation of the growth promoting effects of IGF on these cells (26). In porcine vascular smooth muscle cells, IGFBP-5 potentiated the positive effect of IGF signaling on DNA synthesis, whereas IGFBP-4 had an inhibitory effect on IGF action (35). In a mouse model of prostate cancer, upregulation of IGFBP-5 following androgen withdrawal by castration was found to potentiate IGF signaling *in vivo*, which led to faster progression to androgen dependence (95).

EMERGING ROLE OF IGFBP-5 AS A MOLECULAR SWITCH THAT TURNS ON OR OFF IGF SIGNALING

As discussed above, IGFBP-5 has been shown to be able to inhibit and potentiate IGF signaling in different cell types and/or contexts. When IGFBP-5 was overexpressed *in vivo* in mice, opposite effects were seen on bone formation rate in the periosteum and endosteum suggesting opposite effects on osteoblast proliferation or survival in these regions (100). In vascular smooth muscle cells, IGFBP-5 inhibited IGF-1-dependent DNA synthesis, while it potentiated IGF-1-dependent cell migration (101). How can these seemingly opposite effects be explained? A recent study by Liu et al. (47) has elucidated that while IGFBP-5 inhibits IGF signaling in zebrafish Ca^{2+} transporting ionocytes under normal conditions, it potentiates IGF signaling under low [Ca^{2+}] stress. In addition to IGFBP-5a, these ionocytes highly express Papp-aa, a zebrafish homolog of the IGFBP protease PAPP-A. Treatment of fish with $ZnCl_2$ or batimastat, two metalloproteinase inhibitors (102), inhibited the low [Ca^{2+}] stress-induced ionocyte proliferation, suggesting that Papp-aa protease activity is critical. Genetic deletion of Papp-aa abolished low [Ca^{2+}]-induced ionocyte proliferation, while it had little effect on ionocyte proliferation when fish were kept in normal conditions (47). Loss of Papp-aa expression or activity resulted in diminished IGF1 receptor-mediated Akt-Tor signaling in ionocytes in response to low [Ca^{2+}] stress (47). This phenotype was similar to the igfbp5a$^{-/-}$ mutant fish (88). Biochemically, Papp-aa cleaved IGFBP-5a. Re-introduction of wild-type Papp-aa rescued cell proliferation and IGF signaling, while a protease deficient Papp-aa mutant could not rescue the ionocyte proliferative response (47). Because igfbp5a mRNA levels in each ionocyte did not change under different [Ca^{2+}], Liu et al. (47) speculated that a [Ca^{2+}]-dependent post-transcriptional regulatory mechanisms must block Papp-aa proteolytic activity when [Ca^{2+}] is sufficient (**Figure 4**). This idea was supported by the fact that treatment of fish with NBI-31772, an aptamer that can displace and release IGF from the IGF/IGFBP complex (103) promoted ionocyte proliferation under normal [Ca^{2+}]. NBI-31772 treatment significantly also increased the levels of phospho-Akt and phospho-pS6 activity in ionocytes (47). These data suggest that latent IGF is present and that

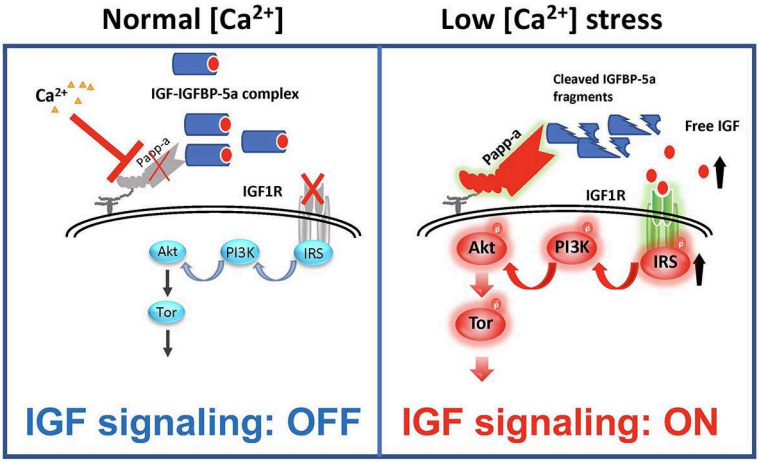

FIGURE 4 | IGFBP-5 is part of a molecular switch that turns IGF signaling on or off in target cells. Zebrafish IGFBP-5a and the conserved zinc metalloproteinase Papp-aa are expressed in ionocytes. Left panel: under normal [Ca^{2+}] conditions, Papp-a proteolysis activity is low. Igfbp5a is intact and it inhibits IGF signaling in these cells by binding to IGFs and prevents their binding to the IGF1R in ionocytes. Right panel: under low [Ca^{2+}] conditions, Papp-a activity is increased. This increases IGFBP-5a proteolytic cleavage and releases IGFs from the IGFBP-5a/IGF complex to activate IGF-1 receptor-mediated PI3 kinase-Akt-Tor signaling and promotes ionocytes to proliferate.

the limiting step under normal [Ca^{2+}] is the release of bioavailable IGFs.

Bony fish produce a hormone called stanniocalcin (STC) in response to high serum [Ca^{2+}] and it inhibits Ca^{2+} uptake (104). The mammalian STC homologs STC-1 and STC-2 were found to strongly inhibit PAPP-A proteolytic activity (105, 106). In zebrafish, the levels of STC-1 mRNA are regulated by Ca^{2+} levels (107). Although it remains to be determined whether endogenous zebrafish STC1 regulates Papp-aa activity in ionocytes, over expression of STC-1 and STC-2 in ionocytes in zebrafish inhibited Papp-aa-dependent activation of ionocyte proliferation (47). Based on these findings, it was postulated that Papp-aa-mediated IGFBP-5a proteolysis functions as a [Ca^{2+}]-regulated molecular switch to conditionally activate IGF signaling in ionocytes (**Figure 4**). Under normal [Ca^{2+}] conditions, Papp-a proteolysis activity is inhibited and Igfbp5a is mostly intact. The intact IGFBP-5a inhibits IGF action by binding to IGFs and preventing their binding to the IGF1 receptor. Under low [Ca^{2+}] conditions, however, Papp-a activity is increased, possibly due to changes in STC1 levels. This increases IGFBP-5a proteolytic cleavage and releases IGFs from the Igfbp5a/IGF complex. This in turn activates IGF-1 receptor-mediated PI3 kinase-Akt-Tor signaling and promotes ionocyte proliferation (47).

IGF-INDEPENDENT ACTIONS OF IGFBP-5

A number of reports have suggested that IGFBP-5 can act via IGF-independent mechanisms (**Figure 2D**). As discussed above, overexpression of IGFBP-5 in mice resulted in significant prenatal and postnatal whole body growth inhibition, which is consistent with the idea that IGFBPs inhibit IGF signaling by inhibiting IGF-IGF1R binding (66). However, overexpression of an IGFBP-5 mutant that lacks binding affinity for IGF also produced significant inhibition of growth, despite the lack of any effects on the IGF signaling pathway (108). This supports the notion that that IGFBP-5 can inhibit growth via an IGF-independent mechanism. Based on *in vitro* studies, IGFBP-5 has been suggested as a bone growth factor and exerts biological activities that are independent of IGFs (109). A number of early reports have suggested that IGFBP-5 binds to its own cell surface receptor, and indeed, IGFBP-3, the most closely related paralog of IGFBP-5, has been found to interact functionally with the type V transforming growth factor beta receptor (LRP-1), which may mediate the IGF-independent growth inhibitory effect (110–112). IGF signaling is crucial for skeletal growth, and both IGF-1 and IGFBPs, including IGFBP-5 are expressed in bone tissue. Binding of IGFBP-5 to sites on the bone cell surface was found to increase proliferation even in the presence of IGF analogs that have 100-fold reduced binding affinity for IGFBPs, suggesting that IGF binding was not required for this action (73). It was suggested that IGFBP-5 may bind to specific receptors on the surface of osteoblastic cells (113). However, to date, no specific IGFBP-5 cell surface receptor has been identified molecularly. But functional interactions with some cell surface proteins have been reported. IGFBP-5 interacted directly with alpha2beta1 integrin on human breast cancer cells *in vitro* and promoted survival and adhesion but inhibited migration (114). Some IGFBP-5 proteolytic fragments were reported to exert IGF-independent actions in cultured bone cells [(76, 77); 44]. In vascular smooth muscle cells, IGFBP-5 was shown to promote cell migration by an IGF-independent mechanism that was facilitated by binding to cell surface proteoglycans (101).

IGFBP-5 contains a conserved nuclear localization sequence (NLS) motif (115), and was shown to be imported in to the nucleus *in vitro* via an importin beta-dependent pathway (23). In addition to its functional NLS, IGFBP-5 was also found to possess

transactivation activity in cell culture experiments (24, 116). IGFBP transactivation and nuclear localization are also found across species ranging from zebrafish to the cephalochordate amphioxus (117). Regulation of target genes by nuclear IGFBP-5 *in vivo,* and possible physiological roles of this activity have yet to be elucidated, but the conservation of IGFBP nuclear transactivation activity across chordate evolutionary history lends credence to the idea that such physiological roles may exist. A yeast two hybrid screen for nuclear protein-protein interaction partners found that IGFBP-5 interacts with nuclear protein FHL2 *in vitro*, but the physiological relevance of this interaction is unclear (118). IGFBP-5 was also found to interact in the nuclei of osteoblast-like cells *in vitro* with the vitamin D receptor (119). This interaction reduced the cellular response to 1,25-dihydroxyvitamin D3 which normally promotes cell cycle exit, differentiation, and expression of bone matrix proteins in these cells, and this effect was seen only when the cells produced IGFBP-5 endogenously and not when it was added exogenously (119).

IGFBP-5 IN PATHOLOGY AND DISEASE STATES

IGFBP-5 has been found to be altered in various disease states (120–123), providing the possibility of using this protein as a marker of disease progression, and hinting that altered IGFBP-5 expression may have pathophysiological relevance. Altered levels of IGFBP-5 have been detected in many types of cancer. Ding et al. (93) have identified over 20 non-synonymous IGFBP-5 mutations in a variety of cancer cell lines. These include frame-shift and non-sense mutations. Several of them, including G223R and W242* were speculated to have lost IGF binding ability. IGFBP-5 has been found to indicate a poor prognosis in patients with several types of cancer (124). IGFBP-5 levels are significantly elevated in osteosarcoma cells that exhibit high metastatic potential (125, 126). However, others found that IGFBP-5 expression inhibited osteosarcoma tumor growth and metastasis (78, 127). In gastric cancer, upregulation of IGFBP-5 was found to partially mediate the action of the PBX/Knotted Homeobox 2 tumor suppressor (128). In papillary thyroid carcinoma, IGFBP-5 was reported to promote cell growth, and miR-204-5p, which inhibits growth by suppressing IGFBP-5, was downregulated in these cells (39). In MCF-7 breast cancer cells, IGFBP-5 promoted cell survival and adhesion via an IGF-independent mechanism (114). A genome wide association study found an SNP allele associated with reduced IGFBP-5 expression and this SNP conferred increased susceptibility to breast cancer, which is consistent with the role of IGFBP-5 in mammary gland discussed above (129). IGFBP-5 has been found to both inhibit and promote cancer cell growth *in vitro* (130–135). It is possible that the expression of IGFBP-5 protease(s) may be important for determining the context-specific effects of IGFBP-5.

IGFBP-5 may play a role in the pathogenesis of atherosclerosis, which is a process of inflammatory tissue remodeling within the matrix of the arterial wall that is the top cause of cardiovascular disease and aging-related mortality in humans (136). A cross-sectional case-control study found a positive association between circulating IGFBP-5 levels and coronary heart disease (137). Overexpression of PAPP-A, whose only known substrates are IGFBP-2, -4, and -5, in the arterial smooth muscle of mice enhanced the progression of atherosclerotic lesion development (138). PAPP-A knockout mice are protected from atherosclerosis as well (139). Conflicting results have been found in mouse models in which other components of the IGF system have been manipulated, and there are indications that circulating IGF may be protective rather than pro-atherosclerotic (140). Local IGF signaling plays an important role in atherosclerosis by stimulating the proliferation of vascular smooth muscle cells and their migration into the arterial intima where they contribute to the formation of atherosclerotic plaques (141–143). Local IGF signaling in the arterial wall and in atherosclerotic plaques is regulated by multiple IGFBPs including IGFBP-5 (101, 144). IGFBP-2 and -4 inhibit IGF signaling in VSMCs but IGFBP-5 enhances it (35, 101). IGFBP-5 expression is upregulated in atherosclerotic plaques and IGFBP-5 protein is found in large quantities associated with ECM within atherosclerotic plaques (56, 145). IGFBP-5 is known to bind to ECM components PAI-1 and osteopontin, which have both been found in atherosclerotic plaques and have been shown to promote atherosclerosis in loss of function studies (28, 146, 147). ECM associated IGFBP-5 potentiates IGF signaling, and IGF signaling can upregulate expression of IGFBP-5, so it is possible that a positive feedback loop could contribute to atherogenesis (28, 143).

IGFBP-5 was shown to be upregulated in lung tissue from patients with idiopathic pulmonary fibrosis (IPF), and exogenous IGFBP-5 also stimulates the secretion of ECM components by IPF lung fibroblasts (148). This effect was independent of IGF-binding but also did not require translocation into the nucleus (149). Exogenous and endogenously expressed IGFBP-5 was found to increase the expression of ECM component genes and pro-fibrotic genes in primary human IPF fibroblasts *in vitro* (150). IGFBP-5 was also shown to increase expression of its own gene in these cells, leading to a positive feedback loop that may play a role in IPF pathogenesis (150).

The role of IGFBP-5 in both atherosclerosis and fibrosis may be linked to the induction of cellular senescence (145, 151). Aged artery walls are more susceptible to atherosclerosis and hypertension which may be related to accumulation of senescent cells and the resultant compositional changes in the subendothelial matrix (136). The accumulation of senescent cells in the arteries of children with the premature aging disease Hutchinson-Gilford Progeria seems to be the cause of their severe accelerated atherosclerosis and premature death from resulting stroke or heart attack before age 20 (152, 153). Senescent cells exhibit a senescence-associated secretory phenotype that is characterized by excessive production of ECM components, and this may play a role in tissue fibrosis (122). IGFBP-5 was upregulated in senescent human umbilical vein endothelial cells and knock down of IGFBP-5 partially reversed the senescence, suggesting a role for IGFBP-5 in promoting cellular senescence (145). The accumulation of senescent cells may play a causal role in many aspects of the vertebrate aging process, which is

known to be promoted by IGF signaling (136). Future research will determine the extent to which IGFBP-5 may be involved in linking IGF signaling to aging-related changes in tissues and the pathology of aging related diseases.

CONCLUSIONS AND PROSPECTS

IGFBP-5 is a multifunctional protein that is capable of regulating IGF signaling both positively and negatively in different tissues and cells. It can also promote, or inhibit cell survival, proliferation, migration, etc. via mechanisms independent of IGF binding. The range of reported IGFBP-5 actions in different cell types can be daunting to understand. There are several possible explanations for the plethora of IGFBP-5 activities: (1) many actions of IGFBP-5 have been reported only in immortalized cell lines *in vitro*, and as such, they can only be accepted as potential actions with uncertain physiological relevance until they are confirmed *in vivo*; (2) different study methodologies may demonstrate opposite findings as result of a downstream effects depending sensitively on the dose of IGFBP-5, i.e., a small amount IGFBP-5 may potentiate IGF signaling while a large enough excess of IGFBP-5 may switch to inhibition; (3) in some cases, exogenous and endogenous IGFBP-5 may act through different mechanisms, possibly as a result of different posttranslational modifications, etc. (79); and (4) IGFBP-5 can act as the pivot point in a switch between regulated states of inhibition and activation of downstream signaling (**Figure 4**). For instance, IGFBP-5a inhibits IGF signaling in zebrafish ionocytes under normal physiological medium, while it potentiates IGF signaling in the same cells when it is proteolytically cleaved by Papp-aa under low [Ca^{2+}] stress (47).

We will not understand why IGFBP-5 has IGF-independent actions until these actions are fully elucidated *in vivo*. However, it is worth considering that some of these actions may have arisen as a result of the opportunistic nature of evolution. If an ancestral IGFBP was originally involved mainly in conditionally regulating the availability of IGFs to their receptors, then the context-specific inducible expression and secretion of IGFBP-5 would have presented a cue that could easily be coopted by evolution in order to trigger other adaptive responses to those same conditions. It is also worth considering that the one IGFBP gene present in the genome of amphioxus contains a nuclear localization sequence and transactivation activity, possibly indicating an ancestral role for IGF-independent functions (117). Further studies are needed in order to determine the circumstances in which this activity may play a role *in vivo*.

The paradox of IGFBPs in general, and IGFBP-5 in particular, is that they each seem to have many unique and important roles, and yet, loss of function experiments in model organisms have generally found either no phenotype or very minimal phenotypes when IGFBPs are deleted (11). This is especially puzzling for IGFBP-5 because it is the most evolutionarily conserved among all of the IGFBPs and yet IGFBP-5 knockout mice had normal growth, organ weights and body composition, and the only reported phenotype was a delay in mammary gland involution (67). But despite the apparent dispensability of IGFBP-5, no vertebrate species is known to have lost this gene. The emerging explanation for this apparent paradox is that IGFBP-5 acts mainly as conditional modulator of IGF signaling which confers an evolutionary advantage by facilitating the rapid adaptation of cell population growth rates to the needs of the environment. It is possible that we are not aware of all of the specific cases in which IGFBP-5 may conditionally act in different species. It is expected that these cases would not arise in laboratory conditions but would be much more likely to occur in response to the vicissitudes of life in the natural environment. The requirement of zebrafish IGFBP-5a for survival under low [Ca^{2+}] stress (88) provides a paradigmatic example of the kind of circumstances in which previously undiscovered IGFBP-5 functions may be found. Future studies will clarify whether there are in fact other sets of conditions in which IGFBP-5 activity is required for survival.

AUTHOR CONTRIBUTIONS

CD conceived this review. CD and JA wrote this review.

FUNDING

Research in the Duan laboratory was supported by NSF Grants IOS-1051034 and IOS-1755268.

ACKNOWLEDGMENTS

The authors apologize to those researchers whose work was not cited in this article due to limitations of space and scope.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

CORRECTION
published: 28 August 2020
doi: 10.3389/fendo.2020.00597

Corrigendum: Insulin-Like Growth Factor Binding Protein-5 in Physiology and Disease

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Keywords: IGF signaling, AKT, mTOR, PAPP-A, STC, IGF-dependent, IGF-independent action

OPEN ACCESS

Approved by:
Frontiers Editorial Office, Frontiers Media SA, Switzerland

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Specialty section:
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *20 July 2020*
Accepted: *22 July 2020*
Published: *28 August 2020*

Citation:
Duan C and Allard JB (2020) Corrigendum: Insulin-Like Growth Factor Binding Protein-5 in Physiology and Disease. Front. Endocrinol. 11:597. doi: 10.3389/fendo.2020.00597

A Corrigendum on

Insulin-Like Growth Factor Binding Protein-5 in Physiology and Disease
by Duan, C., and Allard, J. B. (2020). Front. Endocrinol. 11:100. doi: 10.3389/fendo.2020.00100

An author name was incorrectly spelled as **Cumming Duan**. The correct spelling is **Cunming Duan**.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
published: 03 March 2020
doi: 10.3389/fendo.2020.00024

The IGF/Insulin-IGFBP Axis in Corneal Development, Wound Healing, and Disease

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular and Structural Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *01 October 2019*
Accepted: *14 January 2020*
Published: *03 March 2020*

Citation:
Stuard WL, Titone R and Robertson DM (2020) The IGF/Insulin-IGFBP Axis in Corneal Development, Wound Healing, and Disease. Front. Endocrinol. 11:24. doi: 10.3389/fendo.2020.00024

The insulin-like growth factor (IGF) family plays key roles in growth and development. In the cornea, IGF family members have been implicated in proliferation, differentiation, and migration, critical events that maintain a smooth refracting surface that is essential for vision. The IGF family is composed of multiple ligands, receptors, and ligand binding proteins. Expression of IGF type 1 receptor (IGF-1R), IGF type 2 receptor (IGF-2R), and insulin receptor (INSR) in the cornea has been well characterized, including the presence of the IGF-1R and INSR hybrid (Hybrid-R) in the corneal epithelium. Recent data also indicates that each of these receptors display unique intracellular localization. Thus, in addition to canonical ligand binding at the plasma membrane and the initiation of downstream signaling cascades, IGF-1R, INSR, and Hybrid-R also function to regulate mitochondrial stability and nuclear gene expression. IGF-1 and IGF-2, two of three principal ligands, are polypeptide growth factors that function in all cellular layers of the cornea. Unlike IGF-1 and IGF-2, the hormone insulin plays a unique role in the cornea, different from many other tissues in the body. In the corneal epithelium, insulin is not required for glucose uptake, due to constitutive activation of the glucose transporter, GLUT1. However, insulin is needed for the regulation of metabolism, circadian rhythm, autophagy, proliferation, and migration after wounding. There is conflicting evidence regarding expression of the six IGF-binding proteins (IGFBPs), which function primarily to sequester IGF ligands. Within the cornea, IGFBP-2 and IGFBP-3 have identified roles in tissue homeostasis. While IGFBP-3 regulates growth control and intracellular receptor localization in the corneal epithelium, both IGFBP-2 and IGFBP-3 function in corneal fibroblast differentiation and myofibroblast proliferation, key events in stromal wound healing. IGFBP-2 has also been linked to cellular overgrowth in pterygium. There is a clear role for IGF family members in regulating tissue homeostasis in the cornea. This review summarizes what is known regarding the function of IGF and related proteins in corneal development, during wound healing, and in the pathophysiology of disease. Finally, we highlight key areas of research that are in need of future study.

Keywords: cornea, IGF-1, IGF-1R, INSR, Hybrid-R, IGFBP-2, IGFBP-3

INTRODUCTION

The cornea constitutes the outer covering of the eye and provides two thirds of the refractive power necessary for vision. It is composed of five layers including a stratified epithelium, Bowman's membrane, the collagenous stroma, Descemet's membrane, and the single cell layered endothelium (**Figure 1A**). Corneal innervation is supplied by the ophthalmic branch of the trigeminal nerve. While corneal innervation is described in detail elsewhere and is beyond the scope of this review, it is important to note that the corneal epithelium is the highest innervated structure in the body due to the high density of intraepithelial nerve terminals (**Figure 1B**) (4). This, along with the tight barrier function of the epithelium, plays a major role in protecting intraocular structures from the outside environment. In addition to protection, the cornea must maintain a smooth, transparent, and avascular appearance to allow for the passage of light. The avascular nature of the cornea is one of the cornea's many unique properties. Other important properties include the peripheral location of stem cells in the corneal limbus, the paired movement of daughter cells in the central cornea from the basal layer to the surface epithelium, the epithelium's exceedingly high glycogen content, and the precise organization of collagen lamellae that facilitates transparency.

Corneal wound healing is complex and requires a unique orchestration of events including resurfacing of the corneal epithelium, deposition of basement membrane, and regeneration of the extracellular matrix. At the level of the epithelium, immediately upon wounding, the basement membrane is disassembled and epithelial cells surrounding the wound margin migrate as a sheet to cover the wound. In the presence of an incisional wound, epithelial cells migrate down into the wounded stroma. Once the wounded area is fully covered by these flattened epithelial cells, proliferation of limbal and transient amplifying cells ensues, followed by migration, stratification, the re-establishment of junctional proteins, and the restoration of the basement membrane. When wounds extend beyond the epithelium into the corneal stroma, cytokines released by epithelial cells and present in tears transform the normally quiescent keratocytes into fibroblasts that migrate into the wounded area. This is followed by their sequential transformation into myofibroblasts, strong contractile cells that function to close the wound. Ultimately, over time, keratocytes repopulate, and remodel the extracellular matrix. However, abnormal extracellular matrix often persists, resulting in corneal fibrosis. Unlike the corneal epithelium, when damaged, the corneal endothelium does not proliferate to close a wound. Instead, endothelial cells flatten and spread, taking on a polymorphic appearance.

Many systemic diseases, such as diabetes, can dramatically alter the normal biology of the cornea, resulting in a thinned, dysplastic epithelium with damaged corneal nerves, and cell loss in the endothelial monolayer, the latter of which drives corneal swelling. Most notably, since diabetes can negatively impact all layers of the cornea, impaired corneal wound healing can present a major clinical problem that is often refractory to therapy. While greatly under-recognized, corneal complications occur in 40–70% of diabetics (5). These complications, which range from mild

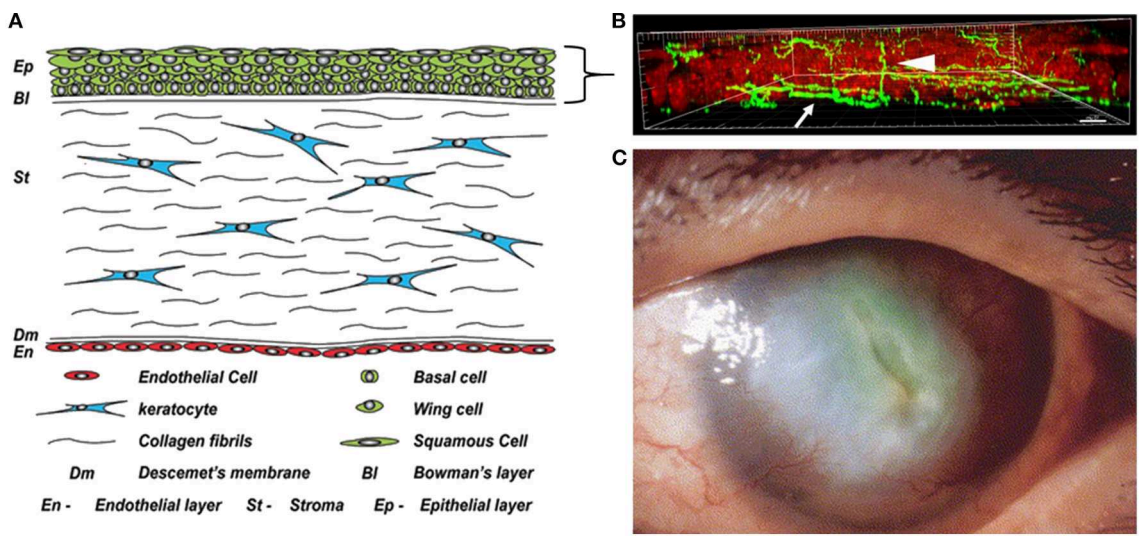

FIGURE 1 | The cornea in health and disease. **(A)** Anatomical schematic showing all five cell layers in the cornea. A five to seven stratified layer of epithelial cells (basal, wing, and squamous) composes the corneal epithelium. Keratocytes, normally quiescent cells, reside in the corneal stroma which consists of intertwining collagen fibrils. Finally, there is a single endothelial cell layer on the innermost layer of the cornea that faces the interior of the eye and is responsible for maintaining stromal hydration. Figure taken from Polisetti et al. (1). **(B)** Maximum intensity projection of nerves labeled with neuronal beta tubulin (green) in the mouse cornea *in situ*. Epithelial nuclei are counterstained with propidium iodide in red. Nerve fibers that run among the basal layer of the epithelium and just beneath it form the subbasal nerve plexus (arrow). Intra-terminal nerve fibers branch perpendicularly from the subbasal nerve plexus and run throughout the corneal epithelium to the surface of the eye (arrowhead). Figure taken from Cai et al. (2). **(C)** A cornea with diabetic keratopathy. Note the large central opacification and significant neovascularization. Image taken from Matsumoto et al. (3).

to severe, can result in chronic and painful corneal complications, predispose the cornea to infection, and in advanced stages, lead to neurotrophic disease and blindness (**Figure 1C**) (6, 7). The importance of proper growth factor signaling in the normal and diabetic cornea is well-established. The focus of this review is to chronicle what is known about the localization and function of insulin-like growth factor (IGF) family members in the cornea and to highlight critical areas of investigation for future studies.

THE IGF SYSTEM

The insulin-like growth factor (IGF) system consists of two peptide ligands, IGF-1 and IGF-2, and the hormone insulin (**Figure 2**). These extracellular ligands activate the IGF Type 1 receptor (IGF-1R), the IGF type 2 receptor (IGF-2), and insulin receptor (INSR), all with varying affinities. The system is further regulated at the extracellular level by the presence of IGF-binding proteins (IGFBPs). There are six known IGFBPs. Historically, IGFBPs function to bind IGF-1 to prolong its half-life in circulation and to prevent IGF-1 induced activation of IGF-1R. This is mediated by proteolytic enzymes that function to cleave IGFBPs, thereby regulating the amount of bioavailable IGF-1. To date, two known proteases have been identified. They include the pregnancy-associated plasma proteins, PAPP-A, and PAPP-A2, which are inhibited by the stanniocalcins (8, 9). In addition, the ligands, as well as binding proteins, have been shown to interact with IGF family receptors to exert unique effects that are cell and tissue dependent (10, 11). IGF-1 is well-known for its role in growth and development in physiologically healthy tissues (12). In the cornea, IGF-1R, IGF-2R, and INSR and their canonical ligands have been studied in the epithelium, stroma, and endothelium. As seen in other tissues, these works have demonstrated key biological roles for members of the IGF family in proliferation, homeostasis, differentiation, and wound healing.

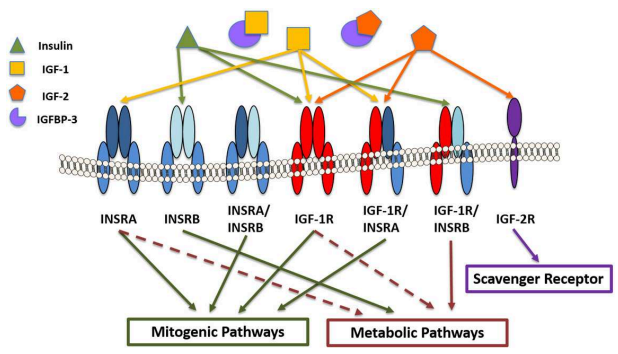

FIGURE 2 | IGF/insulin-IGFBP family members in the corneal epithelium. Receptors include insulin receptor A and B (INSRA and INSRB), IGF type 1 and type 2 receptors (IGF1-R and IGF-2R) and the hybrid receptors (INSRA/INSRB, IGF-1R/INSRA, and IGF1-R/INSRB). Insulin binds with higher affinity to INSRB and the hybrid receptor (IGF-1R/INSRB), predominantly activating metabolic pathways. IGF-1 and IGF-2 bind with higher affinity to IGF-1R and IGF-1R/INSRA, activating mitogenic signaling. IGF-2 also binds the scavenger receptor, IGF-2R. IGF binding protein-3 (IGFBP-3) sequesters IGF-1 and IGF-2, preventing them from binding to their cognate receptors.

CHARACTERIZATION OF IGF-1R, IGF-2R, AND INSR IN THE HUMAN CORNEA

Structurally, IGF-1R and INSR are transmembrane tetrameric glycoproteins comprised of two transmembrane beta subunits, each containing an intracellular tyrosine kinase domain, and two alpha subunits, each containing an extracellular ligand binding domain (13). In contrast to these receptors, IGF-2R is a monomeric transmembrane protein with 15 different extracellular domains (14). INSR differs from IGF-1R and IGF-2R in that it undergoes alternative splicing at exon 11 resulting in two different isoforms, INSRA and INSRB. Each isoform is thought to play distinct roles in development and metabolism. Expression of each also mediates affinity for insulin, kinase activity, and may contribute to the rate of internalization and receptor recycling.

INSR was first identified in the cornea by Naeser in the late 90's (15). In that work, he used immunohistochemical techniques to stain for INSR in donor human corneas with and without diabetic retinopathy. He found strong staining for INSR in the corneal epithelium, stromal keratocytes, and endothelium. Staining was unchanged in diabetes. Rocha and colleagues also used immunohistochemical techniques to show that INSR was indeed expressed in the corneal epithelium (16). In their study, INSR localized to the cytoplasm and plasma membrane primarily in the wing and superficial cell layers. Varied expression was evident in the basal and intermediate suprabasal cell layers. While not reported, INSR appeared to be expressed in nuclei of corneal epithelial cells. More recent work by our laboratory has used multiple complementary approaches to confirm expression of INSR in corneal epithelial cells and to define the intracellular localization of this protein (as discussed in greater detail later in this manuscript).

Nakamura first used ligand-binding assays to confirm the presence of IGF-1R in corneal epithelial cells and showed that IGF-1R had the greatest affinity for IGF-1, followed by IGF-2, and then insulin (17). As they did for INSR, Rocha also stained for the presence of IGF-1R in the corneal epithelium. Using an antibody that recognizes the extracellular alpha subunit of IGF-1R, they demonstrated robust staining at the plasma membrane throughout all epithelial layers. Subsequent studies by our laboratory have further characterized the expression and localization of IGF-1R in cultured corneal epithelia (11, 18, 19). Using antibodies that recognize both the alpha and beta subunits of IGF-1R, we found that the full mature receptor localized to the nucleus of corneal epithelial cells. We confirmed this using subcellular fractionation and immunoblotting assays. We further found that IGF-1R interacts with E-cadherin at areas of cell-cell junctions (18). Interactions between IGF-1R and E-cadherin have been previously reported in tumor biology where they are thought to regulate tumor invasion and in Madin Darby canine kidney cells (20, 21). In this latter cell type, is was shown that binding to E-cadherin negatively regulated ligand-induced activation of IGF-1R (20). The significance of the interaction between IGF-1R and E-cadherin in the corneal epithelium is unknown. Consistent with a growth inhibitory role, we postulate that E-cadherin binding is necessary to attenuate

IGF-1R activation and downstream signaling events that mediate proliferation and growth (18).

It has long been known that IGF-1R and INSR are highly homologous receptors, with 45–65% homology in the amino acid sequence in the alpha subunit and 84% homology in the beta subunit (22). Due to this high level of homology, IGF-1R and INSR can hybridize to form an IGF-1R/INSR hybrid (Hybrid-R). Hybrid-Rs can form with either INSR isoform. Hybrid-R is expressed in the corneal epithelium (19). Using dithiothreitol to cleave the class 1 disulfide bonds and separate IGF-1R alpha/beta subunits from INSR alpha/beta subunits following stimulation with IGF-1 or insulin, we confirmed that Hybrid-R is activated by IGF-1 and not insulin in corneal epithelial cells. Since INSR isoform A is predominantly activated by IGF-1 to promote IGF-1 signaling, whereas INSR isoform B is predominantly activated by insulin and functions to attenuate IGF-1 signaling, our data suggest that in the corneal epithelium, Hybrid-R is composed of IGF-1R and INSR isoform A (19). This finding remains to be confirmed at a protein level.

Bohnsack and colleagues evaluated IGF-2R distribution in human, murine, and porcine corneas. In their study, they found that IGF-2R was present throughout all cell layers of the cornea (23). In murine and porcine models, expression was primarily localized to the basal epithelium. After wounding, they reported an 11-fold increase in IGF-2R in the stroma and epithelium. The increase in IGF-2R was associated with an increase in differentiation of fibroblasts to myofibroblasts, demonstrated by an increase in α-SMA expression, which was subsequently blocked by shRNA knockdown of IGF-2R. Likewise, when keratocytes were cultured in serum free media and treated with TGF-beta to induce myofibroblast differentiation *in vitro,* there was a similar increase in IGF-2R expression in myofibroblasts, at the mRNA and protein level. Together, these findings suggest a potential role for IGF-2R in mediating fibroblast to myofibroblast transformation during wound healing.

INTRACELLULAR IGF-1R, INSR, AND HYBRID-R

More recently, non-canonical roles for IGF-1R and INSR have been suggested (24). In their seminal paper, Sehat et al. used human melanoma (DFB) and leiomysocarcoma (SKUT-1) cells along with human embryonic kidney cells (HEK 293) to first describe the nuclear localization of IGF-1R (25). Using a serum-based model, they demonstrated that serum starvation depletes IGF-1R from the nucleus and that treatment with IGF-1 induced translocation of IGF-1R from the plasma membrane to the nucleus. They further showed that translocation to the nucleus was mediated by SUMOylation of IGF-1R by the SUMO modifier SUMO-1. It has since been shown that nuclear IGF-1R interacts with several different nuclear proteins and functions to regulate the cell cycle, DNA damage responses, invasion, and metastasis (26–29).

Like IGF-1R, studies have also reported that INSR localizes to the nucleus. In the late 70's, Goldfine and colleagues were the first to demonstrate that insulin bound to isolated nuclei *in vitro* (30). They further showed that treatment of isolated nuclei with trypsin prevented insulin binding. The authors concluded that a hormone receptor modulated by insulin was present in rat liver nuclei. Just over a decade later, a second group refuted these earlier findings. In their study, Soler et al. used a combination of *in vitro* techniques to investigate a potential nuclear localized receptor (31). In contrast to the prior work, they concluded that once dissociated from INSR, the biologically intact and active hormone accumulated in the nucleus and associated with heterochromatin. Until recently, few studies have followed up on the potential for a nuclear-localized INSR. In rat hepatocytes, INSR has been shown to translocate to the nucleus where it regulates calcium signals and proliferation (32). Hancock and colleagues have also investigated the role of INSR in the nucleus of mouse liver cells (33). In their studies, they found that INSR directly associated with genome-wide promoters and regulates gene expression through interactions with RNA polymerase II.

Consistent with these studies, we have found that both IGF-1R and INSR localize to the nucleus of corneal epithelial cells (18). Unlike prior studies however, we have found that the nuclear localized receptor is Hybrid-R (19). We have further shown that Hybrid-R nuclear translocation occurs in response to growth factor withdrawal and is not induced by stimulation with IGF-1. Instead, expression and localization of each receptor is mediated by insulin (11). In the absence of insulin, expression of IGF-1R and INSR is upregulated and the receptors accumulate as Hybrid-R in the corneal epithelial cell nucleus. This is mediated through SUMOylation by the SUMO modifier SUMO2/3. The ability of insulin levels to regulate Hybrid-R nuclear translocation is due to its ability to regulate extracellular levels of IGFBP-3. Studies in our laboratory which decrease expression of IGFPB-3 using siRNA knockdown followed by the addition of exogenous recombinant human IGFBP-3 not only demonstrate robust translocation to the nucleus, but also drive receptor accumulation in the insoluble nuclear fraction, indicating association with DNA (11). While we have been unable to detect the presence of IGF-1R alone in the nucleus, we have not yet ruled out the presence of INSR not complexed with IGF-1R.

Our more recent studies on the function of intracellular IGF-1R, INSR, and Hybrid-R have led to the novel observation that INSR and IGF-1R are present in mitochondria (34). Using mitochondrial fractionation assays, we have confirmed that IGF-1R and INSR localize to mitochondria and that expression of both accumulates during stress induced by growth factor withdrawal. Using reciprocal immunoprecipitation, we have further found that INSR and IGF-1R bind the voltage gated anion channel, VDAC1. Importantly, when we disrupt the interaction between INSR and VDAC1 using INSR knockdown, we see robust mitochondrial ring/donut shaped fragmentation. This finding indicates that the novel interaction between INSR and VDAC1 is important for mediating mitochondrial stability (34).

INSULIN AND GLUCOSE UPTAKE

Insulin is a peptide hormone that functions to mediate metabolic effects in addition to growth and proliferation. Structurally,

bioactive insulin presents in humans as a monomer consisting of two chains: an A-chain and B-chain, joined by disulfide bonds (35, 36). Insulin is produced by beta cells in the pancreas, where it is stored and secreted in response to high levels of blood glucose (37). In most cell types and tissues, insulin is required for glucose uptake. In tissues such as skeletal muscle, adipose tissue, and lungs, insulin binds INSR and promotes the uptake of glucose from circulation through glucose transporter-4 (GLUT4) (38). In certain tissues however, such as the corneal epithelium, glucose uptake is insulin independent, meaning that corneal epithelial cells do not require insulin for glucose uptake (**Figures 3A,B**) (39, 40). Instead, glucose uptake is mediated by a constitutively active glucose transporter, GLUT1 (41). This allows for the continuous passage of glucose into cells (39). In conditions where the metabolic demand is increased, such as following a wound, the corneal epithelium responds by increasing the number of GLUT1 transporters in order to provide sufficient energy for proliferation, migration, and survival (42).

Using a rat corneal wound model, Takahashi and colleagues found that levels of both GLUT1 mRNA and protein were increased in the corneal epithelium as early as 4 h after wounding, peaking at 2 days post-injury (43). They hypothesized that the increase in GLUT1 was necessary to facilitate increased glucose uptake and provide fuel to promote wound healing. In a subsequent study by the same group, they used streptozotocin, a drug known to kill insulin-producing pancreatic beta cells, to induce Type 1 diabetes mellitus. They then examined GLUT1 expression before and after wound healing. They found that in response to the wound, GLUT1 was similarly increased in both diabetic and control groups compared to the non-wounded controls. However, there was no difference in receptor expression prior to wounding between diabetic and non-diabetic animals. Together, their findings indicated that GLUT1 expression had no impact on delayed corneal wound healing in diabetes. This work is in agreement with early studies done by Kumagai et al. that also failed to show any

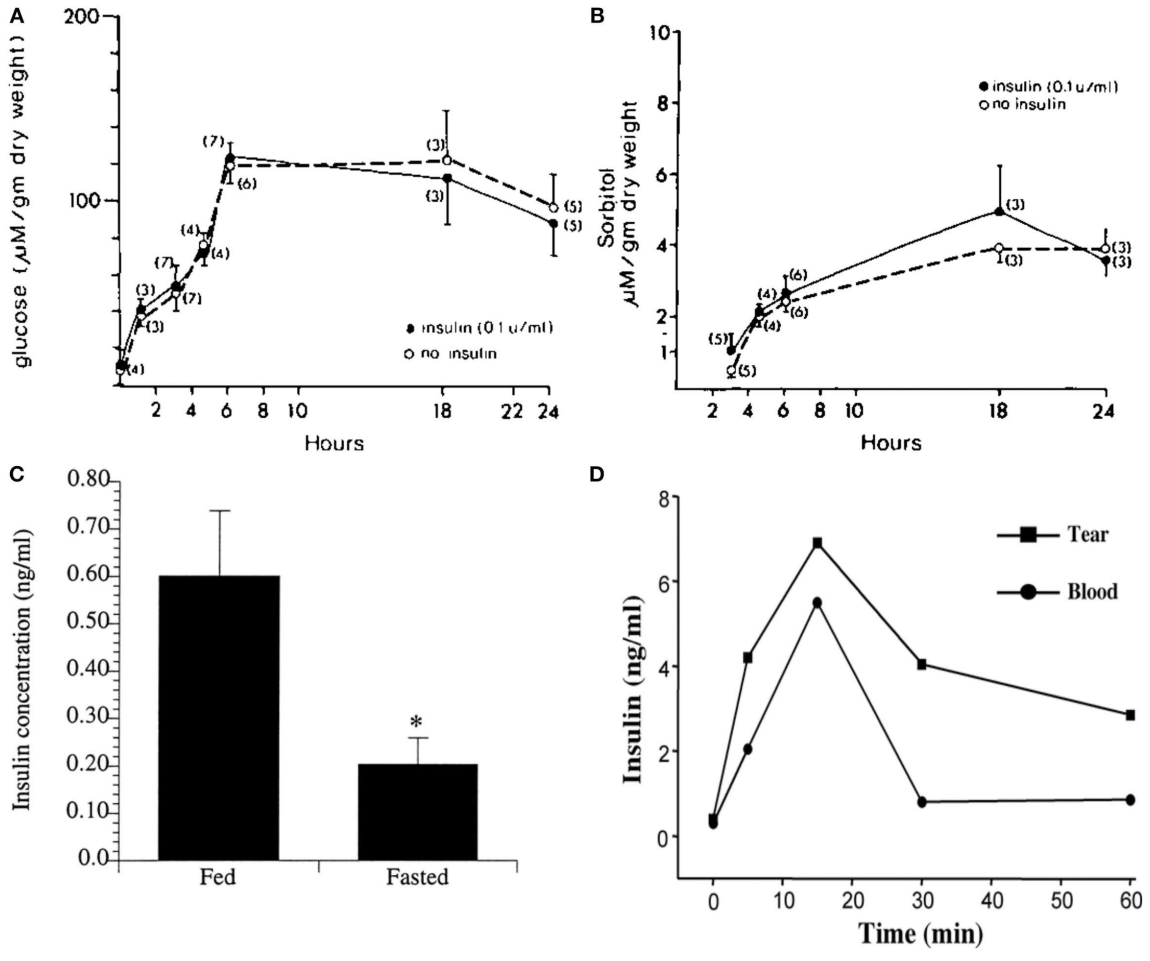

FIGURE 3 | Corneal epithelial cells are "insulin insensitive" and do not require insulin for glucose uptake. **(A)** Glucose content (micromoles per gram ± SEM) of corneal epithelial cells after incubation with 35 mM glucose in TC-199 with and without insulin. **(B)** Corneal epithelial cell sorbitol (micromoles per gram ± SEM) content after incubation with 35 mM glucose in TC-199 with and without insulin. A&B adapted from Friend et al. (39). **(C)** Radioimmunoassay (RIA) measurement of mean insulin levels present in tears of fasted vs. fed human subjects, $P < 0.05$. Adapted from Rocha et al. (16). *$P < 0.05$. **(D)** Insulin levels in tears measured by RIA over time after systemic administration of glucose (1 g/kg body wt) in rats. Adapted from Cunha et al. (40).

differences in GLUT1 expression in the diabetic vs. non-diabetic human cornea (44).

In addition to its role in glucose homeostasis, insulin has broader cellular functions including regulation of cell metabolism, autophagy, apoptosis, growth, and proliferation (45). While insulin is not required for glucose uptake, insulin and its receptors are present in the human cornea and tear film (16). Moreover, insulin levels are increased in tears in fed individuals compared to fasted (**Figure 3C**). Similar to humans, insulin is increased in the rat tear film following a single bolus of glucose administered intravenously (**Figure 3D**) (40). It is unknown whether tear derived insulin is taken up by terminally differentiated surface epithelial cells or is able to somehow cross the tight epithelial barrier, the latter of which is unlikely. Thus, the functional significance of insulin in tear fluid is unknown.

INSULIN AND METABOLISM IN CORNEAL EPITHELIAL CELLS

Cunha and colleagues were the first to confirm a role for insulin in corneal metabolism (40). Studies in our laboratory have sought to further this work and define the mechanism by which insulin regulates cellular metabolism and growth in the corneal epithelium. To accomplish this, we first investigated the role of insulin in regulating cell cycle control in human corneal epithelial cells. After 48 h of growth factor withdrawal, corneal epithelial cells arrested in G0/G1. This arrest was partially restored following treatment with insulin for the final 24 h (11). In that same study, we determined the metabolic phenotype of corneal epithelial cells. We again found that insulin was able to partially restore mitochondrial respiration. This was not due to a shift in glycolysis however, but an increase in mitochondrial respiration (11). To further investigate these findings, we tested the effect of co-treatment with insulin when cells were cultured in basal conditions (no growth factors). Interestingly, we found that corneal epithelial cells undergo a metabolic fuel switch between 24 and 48 h of culture during growth factor withdrawal. In the first 24 h, metabolic activity is driven principally by mitochondrial respiration, whereas in the last 24 h, glycolysis is upregulated to account for a sudden decrease in respiration. In both conditions, insulin was able to maintain both respiration and glycolysis. Consistent with the measured drop in respiration at 48 h, fluorescent imaging showed that mitochondria were largely depolarized. Similar to its effect on respiration, co-treatment with insulin also blocked the loss of depolarization in these cells (**Figure 4**) (34). Together, these findings support that insulin promotes mitochondrial respiration in corneal epithelial cells by maintaining mitochondrial polarization.

Insulin is known to activate PI3K/Akt/mTOR signaling by first binding INSR or IGF-1R at the plasma membrane and then activating downstream cell survival pathways (46). In our laboratory, we showed that insulin regulates phosphorylation of Akt at ser473 in human corneal epithelial cells (11). Activation of this kinase cascade led to an increase in phosphorylation of GS3Kβ at the inhibitory residue, ser9. Since GS3Kβ is a key regulator of cell cycle control, mitochondrial function and apoptosis, and the autophagy inhibitor mTOR, phosphorylation of this residue leads to activation of mTOR and a block in autophagy (47, 48). Autophagy or macroautophagy is a cannibalistic mechanism used by cells to recycle damaged components and debris (49). Selective autophagy represents organelle-specific autophagy. Mitophagy, which is a key mitochondrial quality control mechanism, is the process whereby mitochondria are targeted to autophagosomes (50). In the presence of mitochondrial depolarization, as shown in our growth factor withdrawal model, PTEN-induced kinase 1 (PINK1) becomes stabilized in the mitochondria. PINK1 is a well-studied mitophagy marker that functions to recruit Parkin to the mitochondria. Parkin in turn ubiquitinates mitochondrial proteins, triggering recognition by the autophagosome for subsequent engulfment by the autophagolysosome. Similar to autophagy, insulin blocks all autophagic flux, including mitophagy, in corneal epithelial cells (34). In contrast to this, in breast cancer cells, IGF-1 has been shown to induce mitophagy through activation of the mitophagy receptor BNIP3 (51). Whether IGF-1 is able to activate macro- or selective autophagy in corneal epithelial cells is still unknown, but represents an exciting avenue for study.

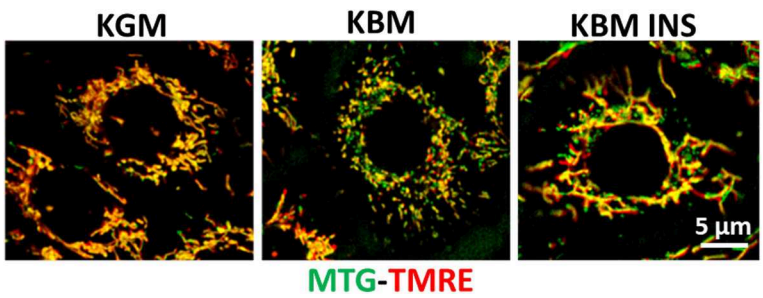

FIGURE 4 | Insulin blocks loss of mitochondrial depolarization. hTCEpi cells were cultured for 48 h in KGM or KBM with or without 5 µg/ml of insulin. Mitochondria were stained with MitoTracker green (MTG, green), a marker of mitochondrial morphology, and TMRE (red), a marker for polarized mitochondria. Culture in basal media without growth factor supplements resulted in mitochondrial depolarization and fragmentation. Concurrent treatment with insulin blocked depolarization and stimulated mitochondrial elongation. KGM, keratinocyte growth media; KBM, keratinocyte basal media; INS, insulin; hTCEpi cells, human telomerized corneal epithelial cells. Scale bar: 5 µm. Adapted from Titone et al. (34).

INSULIN AND THE DIABETIC CORNEA

Several studies have described the effects of high glucose on corneal epithelial homeostasis through modulation of cell signaling, cell proliferation, and wound healing (7, 52–54). Clinically, these changes are manifested in the diabetic cornea in the form of superficial punctate keratitis, alterations in epithelial barrier function, recurrent epithelial erosions due to the presence of an abnormal basement membrane, persistent epithelial defects, and refractory wounds despite treatment (7, 54). In addition to cellular changes, the loss of corneal nerves drives a reduction in corneal sensitivity and this leads to epithelial thinning and reduced tear secretion. The latter of which underlies a cause of dry eye (53). The mechanisms leading to development of corneal complications are multifactorial and are due in part, to abnormal growth factor signaling and the accumulation of reactive oxygen species (53).

Due to the ability of insulin to promote proliferation in cell culture, topical insulin has been proposed as a treatment modality to promote corneal epithelial wound healing (**Table 1**). In their study, Zagon et al. used streptozotocin to induce diabetes in Sprague-Dawley rats (55). In this model, they made a 5 mm corneal wound, followed by treatment with topical insulin four times a day for 7 days. Compared to the vehicle control, topical insulin promoted epithelial resurfacing and an increase in proliferation of basal epithelial cells. Interestingly, treatment with topical insulin also restored corneal sensitivity to normal levels, suggesting that insulin also promoted corneal re-innervation.

Bastion later reported on the results of a retrospective study evaluating the effects of topical insulin on healing epithelial defects in human diabetic patients who were subject to epithelial debridement while undergoing vitreoretinal surgery (56). Fifteen eyes of 14 patients (one patient had bilateral epithelial defects) were divided into one of three groups, diabetics who received topical insulin, diabetics who did not receive topical insulin, and non-diabetics who received topical insulin. In all cases where insulin was administered, re-epithelialization was accelerated compared to non-insulin controls. There were no cases of toxicity or adverse events reported. However, only five patients were evaluated per group. Fai et al. reported on the results of a much larger study (57). Over a 2 year period, all patients with epithelial defects following vitreoretinal surgery were recruited and randomized into one of four groups: 0.5, 1.0, or 2 units of insulin or a saline control. Patients receiving 0.5 units of topical insulin four times a day demonstrated the best efficacy, with clinical effects being lost at the highest dosage.

More recently, Wang and colleagues reported on a small case series of six human subjects with refractory corneal ulcers (59). Patients ranged in age from 2 to 73 years and all presented to clinic with non-healing neurotrophic corneal ulcers. Patients all had past ocular histories that included a battery of therapeutic treatments and surgical procedures that resulted in incomplete healing. In all six cases, one unit of insulin was administered at a frequency of 2–3 times a day and all patients resolved over a period of 7–25 days.

TABLE 1 | Published studies evaluating the role of IGF family ligands in the cornea.

Molecular pathway	Wound repair	References
Insulin	Promotes epithelial resurfacing and proliferation in rodent model.	(55)
	Enhances healing rates in patients with epithelial defects following vitrectomy.	(56)
	Dosed at 0.5 units QID was effective on healing epithelial defects post vitrectomy in diabetic patients without drug toxicity.	(57)
	Aides in wound repair through restoration of circadian rhythm in the corneal epithelium.	(58)
	Promotes healing in neurotrophic corneal ulcers.	(59)
IGF-1	Used with substance P accelerates corneal epithelial cell migration.	(60)
	Used with the substance P-derived peptide (FGML) increases epithelial migration.	(61)
	Used with Substance P promotes epithelial wound healing in an *in vivo* rabbit model.	(62)
	Used with substance P promotes epithelial attachment to fibronectin and Type VI collagen in a rabbit model.	(60)
	Identified the minimum sequence of substance P necessary for a synergistic wound healing effect.	(63)
	Used with substance P restores barrier function.	(64)
	Used with substance P increases wound healing and barrier function in capsaicin injected rat model.	(65)
	Used with FGLM-NH(2) promotes corneal epithelial wound healing in diabetic rats.	(66)
	C domain is the portion necessary for a synergistic wound healing effect with Substance P.	(67)
	Induces corneal epithelial cell migration and increases lamin-5 and β1-integrin expression.	(68)
	Used with FGLM-amide accelerates resurfacing of persistent epithelial defects.	(69)
	IGF-1 peptide sequence SSSR used with FGLM-amide accelerates wound healing.	(70)
	Upregulates IGF-1R expression in corneal epithelial limbal stem cells and drives differentiation during corneal regeneration.	(71)
	Used with substance P increases the rate of re-epithelialization in rabbits post PRK.	(72)
IGF-2	IGF-2R protein expression increases in corneal wound healing in order to regulate keratocyte differentiation to myofibroblasts.	(23)
	Increases along with its receptor in corneal injury. Aids in the proliferation of keratinocytes and synthesis of N-cadherin.	(73)

INSULIN AND CIRCADIAN RHYTHM

In rodent models, the proliferation of basal epithelial cells in the central cornea and epithelial regeneration following a wound are regulated in part by circadian rhythms (74–77). In addition, oscillations in the expression of core circadian genes, including *Clock* and *Bmal1*, have also been reported (74). While diabetes and other metabolic disorders are known to disrupt normal circadian rhythms, Song and colleagues investigated the impact of diabetes on circadian rhythms in the corneal epithelium using a Type 1 streptozotocin mouse model (58). After 2 weeks of disease, there was a significant change in key clock genes, a reduction in basal cell proliferation, and an increase in leukocyte infiltration in the limbal region. These effects were partially restored by the administration of systemic insulin. Since these studies were performed so early on following onset of diabetes, the circadian effects could be measured without the corresponding effects from loss of the subbasal nerve plexus. Thus, it is likely that alterations in the circadian rhythm reflect an early change in diabetes that contributes to disruption of normal homeostasis.

INSULIN AND THE CORNEAL ENDOTHELIUM

The corneal endothelium is a single cell layer on the posterior aspect of the cornea that is flush with the aqueous humor. Function of the corneal endothelium is critical to maintain optimal hydration and stromal transparency. The role of IGF and insulin in the corneal endothelium has not been well-studied. In the bovine corneal endothelium, IGF-1R is ∼25 times more highly expressed than INSR (78). This finding would explain why stimulation of bovine corneal endothelial monolayers with IGF-1 promoted DNA synthesis. While low levels of IGF-1 are able to not only stimulate DNA synthesis, they also induce an upregulation of the proto-oncogene c-fos. Unlike IGF-1, a high concentration of insulin is required to have a similar effect. This is likely due to the ability of insulin to activate IGF-1R at high levels.

Insulin has been shown to play a key role in the regulation of the Na/K-ATPase pump. The Na/K-ATPase pump functions to maintain water balance in the corneal stroma. In diabetes, endothelial cells are highly subject to damage during intraocular surgery and often display a certain degree of pleomorphism even in the absence of surgery (79). Corneal thickening in diabetes is thus due to improper pump function secondary to loss of corneal endothelial cells. In support of this, studies using an alloxan-induced diabetic rabbit model showed that a decrease in NA/K-ATPase activity was associated with an increase in corneal thickness and poor hydration control (80). Like glucose, insulin is present in the aqueous at a much lower concentration than serum. In alloxan-induced diabetes, levels of insulin in the aqueous are further depleted. To determine whether alterations in aqueous insulin levels in diabetes could account for the changes in pump function, Hatou et al. investigated the effect of insulin on Na/K-ATPase activity in mouse corneal endothelial cells *in vitro* (81). Importantly, they found that administration of insulin (0.01–10 μM) to an endothelial monolayer increased the activity of the Na/K-ATPase pump through activation of protein kinase C in a concentration dependent manner (82). It is important to note that these changes were transient in nature. Thus, a chronic insult from either no insulin in Type 1 diabetes or reduced signaling due to insulin resistance, such as that seen in Type 2 diabetes, may contribute to the pathophysiology of diabetes induced corneal endothelial damage.

IGFS IN WOUND HEALING AND REPAIR

IGF-1 and -2 are homologous peptides that modulate cellular proliferation and differentiation throughout the body (83). Activation of their cognate receptors triggers autophosphorylation of their intracellular kinase domain, leading to downstream activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (22, 84, 85). IGF-1 has been well-studied for its important role in cellular migration and proliferation in non-ocular tissues (86). In all three cell layers of the cornea, IGF has been shown to have critical regulatory functions that preserve homeostasis and promote wound repair. In the corneal epithelium, IGF-1 promotes proliferation. This occurs through activation of Hybrid-R and subsequent phosphorylation of Akt (19). IGF-1 was also shown by Lee and colleagues to induce corneal epithelial cell migration and increased expression of Lamin-5 and β1-integrin. These effects were mediated through the PI3K/AKT pathway (68).

Lastly, some data exists to support that IGF-1 also contributes to the differentiation of limbal stem cells into corneal epithelial cells. In their study, Trosan et al. found that after central epithelial debridement in the mouse cornea, IGF-1 and IGF-2 secretion is increased in corneal epithelial cells, while IGF-1R expression was increased in the limbus (71). Interestingly, the increase in IGF-1R expression in the limbus was driven by IGF-1 and promoted differentiation of limbal epithelial cells, evidenced by an upregulation of the cytokeratin K12. A subsequent study with a similar experimental design showed the same effect for IGF-2 (73).

IGF AND SUBSTANCE P

Much of what is known regarding the function of IGF-1 in corneal epithelial wound healing is focused on the interactive role of IGF-1 with the neuropeptide, substance P (**Table 1**). In their *in vitro* studies, Nishida and colleagues showed that IGF-1 administered at a concentration of 10 ng/ml accelerated corneal epithelial cell migration across a wounded rabbit corneal stroma *ex vivo* when used in conjunction with 25 or 50 μg/ml substance P (60). Likewise, IGF-1 together with substance P, promoted corneal epithelial attachment to fibronectin and Type IV collagen. They further showed that this effect was not due to changes in ligand binding sites for IGF-1, but was mediated by interactions between substance P and the Tachykinin receptor, Nrk1 (17, 87). These findings were confirmed in a rabbit model

subject to epithelial debridement using N-heptyl alcohol (62). Additional work by this group induced corneal neuropathy in a rat model by thermocoagulating the ophthalmic nerve that branches from the trigeminal ganglion (64). Using this model they showed that treatment of corneal epithelial wounds with substance P and IGF-1 improves barrier function in the corneal epithelium by promoting wound healing. Subsequent publications by this same group have further elaborated on these key findings and identified the specific amino acid sequences for both substance P and IGF-1 that are responsible for mediating these effects (63, 65–67, 69, 70, 72).

IGF-1 IN TEAR FLUID

IGF-1 is present in tear fluid, although at very low levels (88, 89). In normal healthy conditions, the ratio of IGF-1 to IGFBP-3 is not sufficient to blunt the effects of IGF-1. However, in human diabetic tear fluid, the ratio of IGF-1 to IGFBP-3 is significantly reduced (89). Since IGF-1 binds IGFBP-3 with a greater affinity than IGF-1R, the shift in the IGF-1 to IGFBP-3 ratio is sufficient to sequester IGF-1 and inhibit the ability of IGF-1 to induce phosphorylation of IGF-1R or Hybrid-R (89). The inability of IGF-1 to promote proliferation in the diabetic corneal epithelium may contribute to delayed wound healing. IGF-1 and IGF-2 have both been shown to be upregulated during corneal wound healing. It is not clear whether either of these proteins are upregulated in the diabetic eye.

IGF-1 AND STROMAL KERATOCYTES

The effects of IGF-1 are not restricted to the corneal epithelium, but also play an important regenerative role in the stroma. Corneal keratocytes, the primary cell type in the stroma, are essential for not only maintaining stromal structure but also form an interconnected, communication network within the cornea. It has been shown that IGFs play an important role in regulating formation of this network. Using the IGF-1R inhibitor, picropodophyllin (PPP), Berthaut found that the addition of IGF-1 in concert with PPP blocked both the number of tubules and interconnections formed by corneal fibroblasts cultured on Matrigel loaded with growth factors (90). IGF-1 is also critical in the process of keratocyte differentiation. During inflammation and wounding, stromal cells become activated and induce a differentiation program. Using a co-culture model, Ko et al. showed that both Simian virus 40-transformed human corneal epithelial cells (HCE) and primary cultured corneal fibroblasts secrete IGF-1 (91). Using siRNA knockdown of IGF-1 in HCEs, they further demonstrated that IGF-1 secreted by corneal epithelial cells induces N-cadherin expression, an adherens junction protein, in cultured corneal fibroblasts and that this was most likely regulated by the zinc finger protein, ZEB1. Unlike cancer cells, where the upregulation of N-cadherin is associated with downregulation of E-cadherin and the subsequent epithelial-mesenchymal transition, the increase in N-cadherin in corneal fibroblasts was not associated with changes in any other junctional proteins.

IGF-1 has also been shown to modulate the TGF-beta/SMAD signaling pathway, although the data is conflicting. Sarenac demonstrated that treatment of keratocytes with IGF-1 inhibited differentiation into myofibroblasts by attenuating TGF-beta signaling (92). They concluded that IGF-1 may be a viable therapeutic option to limit fibrosis during corneal wound healing. In contrast to this, Izumi found that IGF-1 stimulated proliferation of myofibroblasts during wound healing without first reverting cells back to their naïve state (93). This increased proliferation of myofibroblasts would further promote fibrosis. Taken together, these findings suggest that IGF-1 may induce differential effects on stromal cells depending on their differentiation status.

IGF-2 IN STROMAL KERATOCYTES

IGF-2 has been shown to play a key role in development of the murine eye (94). The function of IGF-2 has also been investigated in postnatal corneal development. To accomplish this, Kane et al. used keratocytes harvested from bovine and rabbit corneas (95). They measured collagen production and secretion of IGF-2 and IGFBP-2. Striking differences were noted. Rabbit keratocytes, which were proliferative in culture, secreted both Type I collagen and IGF-2. In contrast, bovine keratocytes, secreted IGFBP-2 and not IGF-2. Culture of bovine keratocytes in conditioned media from rabbit keratocytes promoted proliferation and collagen deposition, suggesting that IGF-2 is important in collagen production. Using microarrays, gene expression was next evaluated in keratocytes obtained from mouse neonates and compared to adults. IGF-2 was the most abundant growth factor present. IGF-I and IGFBP-4 were also detected, but were expressed at much lower levels. Interestingly, prior work by this same group showed that IGF-2 was present in the bovine stroma, despite not being secreted by bovine keratocytes (96). They further demonstrated that IGF-2 was capable of inducing keratocyte proliferation without inducing myofibroblasts differentiation. While the source of IGF-2 in the bovine stroma was not determined, IGF-2 appears to be integral to early stromal development.

IGF-1 AND THE CORNEAL ENDOTHELIUM

There has been limited research done on the role of IGF-1 in the corneal endothelium. In embryonic corneal tissue, IGF-1 has been shown to promote DNA synthesis in endothelial cells (97, 98). Feldman and colleagues later used adult bovine corneal endothelium to test the effects of IGF-1 and insulin on DNA synthesis (78). Using BrdU labeling, they found that insulin and IGF-1 were both able to promote DNA synthesis. While both ligands were effective, insulin required much higher concentrations than IGF-1. This was due to the reduced affinity of insulin for IGF-1R. Another study evaluated the effects of IGF-1 on rabbit endothelial cells. In this study, Choi showed that IGF-1 promotes rabbit endothelial cell proliferation through the IRS-1 pathway (99). IGF-1 did not alter collagen production by these cells. Moreover, IGFBP-2 was produced by rabbit endothelial

cells and functioned to sequester IGF-1. It is important to note however, that corneal endothelial cells do not undergo regeneration *in vivo* in cats, non-human primates, or humans. Thus, while IGF-1 promotes DNA synthesis and proliferation on corneal endothelial cells capable of mitosis, the effect of IGF-1 on the human corneal endothelium is relatively unknown.

IGFBPs

IGF-1 and IGF-2 are secreted into the extracellular environment where they are bound to IGFBPs (100). Currently, there are six highly conserved IGFBPs. IGFBPs are found in serum and most extracellular fluids, including the aqueous humor and vitreous (101, 102). Due to the presence of the blood-retinal barrier, the origins of these binding proteins are thought to be tissues within the eye. Several groups have probed for the presence of IGFBP-3 in ocular tissues. Most of these studies have focused on the localization of IGFBP mRNAs. Arnold et al. were the first to investigate the distribution of the IGFBPs in the eye. Using northern blotting, they reported that mRNAs for IGFBP-2 and IGFBP-3 were present in bovine corneas, but their exact distribution was not specified (101).

In the developing chick embryo, the appearance of IGFBP-2 mRNA expression was found to be temporally and spatially controlled (103). Initially noted in the surface ectoderm at embryonic day 3.5 (E3.5), mRNA transcripts were detected in both the corneal epithelium and endothelium, as soon as the cornea began to develop into multilayers (E6). As development neared completion, IGFBP-2 transcripts were evident in all cells throughout the cornea. In a subsequent study, Burren and colleagues were able to confirm the presence of mRNA for IGFBP-2 in the cornea. In the rat eye, they found that IGFBP-2 localized to the basal layer of the corneal epithelium, keratocytes, and endothelium; however, they were unable to detect transcripts for any of the other binding proteins (104). More recently, expression of all six binding proteins was evaluated in a transgenic rat model that over-expressed the renin-2 gene (*REN-2*). The *REN-2* transgenic rat is a model for hypertension characterized by an alteration in the renin-angiotensin system that controls blood pressure (105). Type 1 diabetes was induced in this model using streptozotocin. The authors reported that transcripts for IGFBP-1, IGFBP-5, and IGFBP-6 were present in the cornea, with IGFBP-5 and -6 found to be expressed at the protein level throughout the cornea, including the corneal epithelium. Moreover, transcript levels for these two binding proteins were altered in diabetes, with IGFBP-5 levels increasing and IGFBP-6 levels decreasing.

IGFBP-2 AND STROMAL FIBROBLAST DIFFERENTIATION

IGFBP-2 has been found to have an important role in mediating differentiation of corneal fibroblasts (102). In this study, the authors demonstrated that human corneal keratocytes express high levels of IGFBP-2. In corneal fibroblasts cultured on plastic, increased levels of IGFBP-2 were associated with increased expression of aldehyde dehydrogenase (ALDH1A1) and keratocan, markers for quiescent keratocytes. In contrast, keratocytes cultured on plastic and treated with TGFβ transformed into myofibroblasts and expressed high levels of α-smooth muscle actin (α-SMA) and very low levels of IGFBP-2. This finding is consistent with the observation that TGFβ downregulates IGFBP-3 in dermal keratinocytes (106). Importantly, co-treatment of myofibroblasts with IGFBP-2 partially blocked this transformation through an increase in ALDH1A1, keratocan, and a partial loss of stress fibers, while siRNA knockdown of IGFBP-2 increased α-SMA. Collectively, these data indicate that IGFBP-2 may be a crucial protein that regulates the sequential transition of keratocytes into fibroblasts and myofibroblasts and provide further support of a critical role for the IGF system in corneal wound healing.

IGFBP-2 AND PTERYGIUM

Recent data has shown a link between IGFBP-2 and human malignancies including prostate, ovarian, and colon cancer (107–109). Similarly, IGFBP-2 has been linked to pathological processes in the cornea. Pterygium, a non-cancerous conjunctival overgrowth onto the cornea, is known to express cellular markers that reflect increased proliferation and cellular invasion. Using cDNA microarrays, IGFBP-2 expression was increased in fibroblasts cultured from the pterygium body compared to conjunctival fibroblasts collected from normal tissue (110). In contrast, there were no differences in IGFBP-3 expression. This finding was confirmed at the protein level and suggests that aberrant IGFBP-2 expression may play a role in the development of pterygium.

IGFBP-3

Unlike IGFBP-2, IGFBP-3 is highly regulated at the post-translational level by glycosylation and phosphorylation (111, 112). These post-translational modifications are hypothesized to regulate IGFBP-3s stability and function. In addition, IGFPB-3 is also regulated by synthesis rate and extensive proteolysis (113, 114). Many tissues produce IGFBP-3 locally, where it plays an important role in growth inhibition, including the corneal epithelium (10, 115, 116). IGFBP-3 has also been described as marker for senescence in cancer and in human fibroblasts (117, 118).

In dermal keratinocytes, IGFBP-3 has been shown to function as the main binding protein that interacts with IGF-1 to modulate proliferation (106). Consistent with this, altered expression of IGFBP-3 is associated with the development of psoriatic lesions (119). Izumi and colleagues used human corneal fibroblasts to show that treatment with TGFβ to induce α-SMA expression also upregulated IGF-1 and IGFBP-3 mRNA (93). As already discussed, the increase in IGF-1 has multiple effects including an increase in myofibroblast proliferation and stimulation of collagen production. Prolonged proliferation of myofibroblasts would contribute to excess fibrosis. IGFBP-3 on the other

hand, modulates proliferation of myofibroblasts in an IGF-dependent manner. In the mouse cornea *in vivo*, following photorefractive keratectomy, IGFBP-3 was upregulated and expressed throughout the corneal stroma (93). IGFBP-3 has been shown to bind to certain extracellular matrix proteins and once bound, may alter its affinity for IGF-1. Thus, a potential temporal or spatial gradient in IGF-1 and IGFBP-3 may regulate the degree of fibrosis during corneal wound healing.

More recently, work by our laboratory has begun investigating the role of IGFBP-3 in mediating stress responses in the corneal epithelium. We have shown that IGFBP-3 is upregulated during growth factor withdrawal. This increase in IGFBP-3 is necessary to induce nuclear translocation of Hybrid-R (10). Once in the nucleus, IGF-1R and IGFBP-3 accumulate in the insoluble fraction (10). IGFPB-3 does harbor a nuclear localization sequence and has been shown to traffic to the nucleus in other cell lines and tissues (120). Most available data suggests that nuclear localization is important in regulating apoptosis. The function of nuclear IGFBP-3 in the cornea is unknown.

While IGFBP-3 is necessary to induce trafficking of IGF-1R, loss of IGF-1R in turn downregulates IGFBP-3. Thus, IGFBP-3 and IGF-1R undergo mutual regulation to maintain homeostasis in the corneal epithelium (10). We have also found that IGFBP-3 secretion is increased in response to hypoxia (unpublished observations), and in response to hyperglycemia (89). In agreement with this latter finding, we have reported that human tear levels of IGFBP-3 are similarly increased in patients with diabetes (121). More importantly, the increase in tear levels of IGFBP-3 in Type 2 diabetes correlates with damage to the corneal subbasal nerve plexus (**Figure 5**) (121). What remains unknown is the size of IGFBP-3 present in human tear fluid and whether this is the full-length glycosylated protein or a smaller cleavage fragment.

SUMMARY AND FUTURE DIRECTIONS

The IGF family is responsible for maintaining tissue homeostasis through the regulation of metabolic and/or mitogenic pathways at all cellular levels in the cornea. In addition to their canonical pathways, recent studies have led to the discovery of important intracellular functions in the corneal epithelium. This includes the nuclear translocation of Hybrid-R to the nuclei of human corneal epithelial cells in an IGF-1 independent manner and the ability of Hybrid-R to bind DNA and modulate gene expression. INSR and IGF-1R are also present in mitochondria where they likewise accumulate in the absence of IGF-1. The interaction of IGF-1R and INSR with VDAC1, a protein present in the outer mitochondrial membrane, suggests novel regulatory functions including the trafficking of molecules and ions, mitochondrial stability, and apoptosis. Further interrogation of these interactions may lead to the identification of critical new regulatory mechanism(s) that mediate mitochondrial function and quality control in the cornea and elsewhere in the body. These exciting new findings may also lead to the development of new therapeutic targets aimed at mitigating or preventing complications in patients with diabetes, where mitochondrial dysfunction is a central feature in the pathophysiology of disease.

One of the interesting characteristics that makes the corneal epithelium distinct from other epithelial tissues is the finding that insulin is not required for glucose uptake. Given the avascularity of the cornea, this is not altogether surprising. Insulin does have important regulatory roles in proliferation and cell growth in the corneal epithelium. These findings are not restricted to cell cultures *in vitro*, but extend to animal models and human studies. In diabetes, topical insulin does promote wound healing. The ability of insulin to restore circadian rhythm through *BMAL1* and *CLOCK* genes may facilitate re-epithelialization, as these rhythms may be disturbed in diabetes. One advantage of insulin compared to IGF-1 as a therapeutic option is that IGF-1 is a potent inducer of angiogenesis. While insulin does have angiogenic capabilities, it is not clear from the limited clinical data whether the development of neovascularization from topical use will develop.

In the last few years, several studies have focused on elucidating non-glucose transport functions for insulin. The most recent findings from our laboratory have demonstrated roles for insulin in the regulation of metabolic homeostasis through control of mitochondrial respiration, glycolysis, and autophagy. We have further shown that insulin regulates

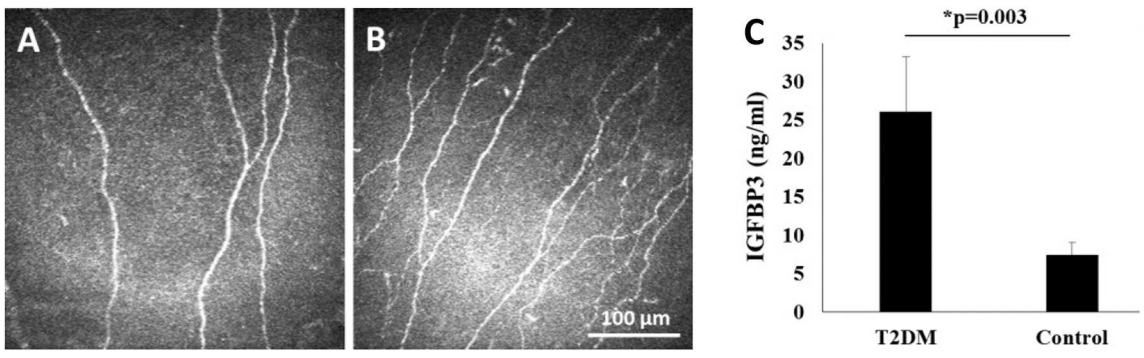

FIGURE 5 | Human tear levels of IGFBP-3 correlate with loss of the subbasal nerve plexus in T2DM. **(A,B)** *In vivo* confocal microscopy of the corneal subbasal nerve plexus showing **(A)** fewer corneal nerve fibers and branches in T2DM; and **(B)** normal nerve morphology in the healthy, non-diabetic control. Scale bar: 100 µm. **(C)** Tear level of IGFBP-3 were increased in patients with T2DM compared to healthy controls ($P = 0.003$, *t*-test). Adapted from Stuard et al. (121).

secretion of IGFBP-3, which in turn, mediates intracellular receptor trafficking. Interestingly, IGFBP-3 secretion is also mediated in part by IGF-1R. Taken together, these findings highlight the crosstalk that occurs between all the components of the IGF system. While the potential presence of proteases that further regulate IGF-1 bioavailability has not yet been investigated, it is clear that there is a delicate balance between members of the IGF-1 family that is critical for normal corneal development and tissue maintenance. Available evidence suggests that this balance is disrupted in diabetes and may contribute in part to recalcitrant wound healing. Moreover, since most of this work has been done in corneal epithelia, the role of insulin in keratocyte and endothelial health is relatively unknown and represents an important area of future study.

IGFBP-3 is a pleiotropic protein whose function is cell and context specific. Based on our prior studies, we hypothesize that IGFBP-3 functions as a major stress response protein in the corneal epithelium. In support of this view, tear levels of IGFBP-3 are increased in patients with diabetes and this increase correlates with loss of the subbasal nerve plexus. Much remains to be done to determine whether or not this discovery will lead to a novel diagnostic test that can be used to monitor patients with diabetes to determine potential risk for neuropathic or ocular complications. The advantages of using tears to monitor patients with diabetes include the ease and the relatively non-invasive nature of collection compared to phlebotomy. However, studies are needed to evaluate the impact of reflex tearing and dry eye on tear levels of IGFBP-3, and to determine its sensitivity and specificity compared to hemoglobin A1c.

In terms of wound healing, the major challenge facing clinicians today is fibrosis. While fibrosis may be disfiguring in skin, it is a leading cause of blindness in the cornea. In severe cases, fibrosis necessitates full thickness corneal grafts to restore vision. There is a growing body of evidence to indicate that IGF family members play an important role in fibrosis. This includes regulating the differentiation of keratocytes into fibroblasts and myofibroblasts and the induction of myofibroblast proliferation without reverting cells back to a fibroblastic phenotype. Much more data is needed to fully understand the contribution of this system to wound healing, the critical crosstalk amongst the differing cell layers in the cornea, and corneal development.

In conclusion, a new outline regarding the impact of IGF family members on the cornea is beginning to emerge. Huge gaps in knowledge persist, creating multiple new areas of much needed research. Future studies will not only allow us to fill in these gaps, but will also allow us to gain a greater appreciation for the function of insulin, IGF, related binding proteins, and proteases, in the normal cornea and in disease.

AUTHOR CONTRIBUTIONS

WS and RT wrote the manuscript. DR wrote and edited the manuscript.

FUNDING

NIH/NEI R01 EY024546 (DR), R01 EY029258 (DR), P30 EY030413 (DR), A Fight for Sight Postdoctoral Fellowship (RT), and an unrestricted grant from Research to Prevent Blindness, New York, NY. The funders had no role in the development/writing of this manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 May 2020
doi: 10.3389/fendo.2020.00233

IGFBP-1 Expression Promotes Tamoxifen Resistance in Breast Cancer Cells via Erk Pathway Activation

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Insulin-like growth factor (IGF) system plays a significant role in many cellular processes, including proliferation, and survival. In estrogen receptor positive breast cancer, the level of circulating IGF-1 is positively associated with the incidence and at least 50% of cases have elevated IGF-1R signaling. Tamoxifen, a selective estrogen receptor modulator and antagonist for estrogen receptor alpha (ERα) in breast tissue, is a commonly prescribed adjuvant treatment for patients presenting with ERα-positive breast cancer. Unfortunately, tamoxifen resistance is a frequent occurrence in patients receiving treatment and the molecular mechanisms that underlie tamoxifen resistance not adequately defined. It has recently been reported that the inhibition of IGF-1R activation and the proliferation of breast cancer cells upon tamoxifen treatment is mediated by the accumulation of extracellular insulin-like growth factor binding protein 1 (IGFBP-1). Elevated IGFBP-1 expression was observed in tamoxifen-resistant (Tam^R) MCF-7 and T-47D cells lines suggesting that the tamoxifen-resistant state is associated with IGFBP-1 accumulation. MCF-7 and T-47D breast cancer cells stably transfected with and IGFBP-1 expression vector were generated (MCF7-BP1 and T47D-BP1) to determine the impact of breast cancer cell culture in the presence of increased IGFBP-1 expression. In these cells, the expression of IGF-1R was significantly reduced compared to controls and was similar to our observations in tamoxifen-resistant MCF-7 and T-47D cells. Also similar to Tam^R breast cancer cells, MCF7-BP1 and T47D-BP1 were resistant to tamoxifen treatment, had elevated epidermal growth factor receptor (EGFR) expression, increased phospho-EGFR (pEGFR), and phospho-Erk (pErk). Furthermore, tamoxifen sensitivity was restored in the MCF7-BP1 and T47D-BP1 upon inhibition of Erk phosphorylation. Lastly, the transient knockdown of IGFBP-1 in MCF7-BP1 and T47D-BP1 inhibited pErk accumulation and increased tamoxifen sensitivity. Taken together, these data support the conclusion that IGFBP-1 is a key component of the development of tamoxifen resistance in breast cancer cells.

Keywords: tamoxifen, breast cancer, drug resistance, IGFBP-1, EGFR

OPEN ACCESS

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Specialty section:
This article was submitted to Molecular and Structural Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *31 October 2019*
Accepted: *31 March 2020*
Published: *06 May 2020*

Citation:
Zheng Y, Sowers JY and Houston KD (2020) IGFBP-1 Expression Promotes Tamoxifen Resistance in Breast Cancer Cells via Erk Pathway Activation. Front. Endocrinol. 11:233. doi: 10.3389/fendo.2020.00233

INTRODUCTION

Insulin-like growth factor (IGF) signaling is a complex system that affects almost every organ in the human body via regulation of multiple cellular processes, such as proliferation, survival, mitogenesis, migration, senescence, angiogenesis, and autophagy (1, 2). The IGF system consists of two natural ligands, insulin-like growth factor-1 (IGF-1) and IGF-2; two transmembrane receptors, insulin-like growth factor 1 receptor (IGF-1R) and IGF-2R; and six high affinity IGF binding proteins (IGFBPs) 1-6 (3). The binding of IGF-1 or IGF-2 to IGF-1R results in the activation of tyrosine kinase activity of the receptor (4), which in turn activates phosphatidylinositol 3-kinase (PI3K)-AKT pathway and mitogen-activated protein kinases (MAPK) pathway (5). IGF-2R, on the other hand, acts as a tumor suppressor and directs the degradation of IGF-2 specifically (6). The bioavailability and half-life of IGF-1 and IGF-2 are tightly regulated by IGFBP1-6 (7), each of which has different binding affinities and distinct functions depending on the tissue (8). In addition to the complexity of the IGF system, there is an increasing body of evidence showing the interactions between IGF pathway and other hormone signaling pathways suck as estrogen receptor (ER) pathway (9) and epidermal growth factor receptor (EGFR).

The IGF system plays an important role in breast cancer as exemplified both *in vitro* and *in vivo* (10). At least 50% of breast tumors present with activated IGF-1R (11) and the level of circulating IGF-1 positively correlates with the incidence of estrogen receptor positive (ER positive) breast tumors (3). The tumor volume was significantly higher in the xenografts containing ER positive MCF-7 cells with IGF-1 overexpression compared to the control in the mouse model (12); IGF-1 potentiated the invasive ability of MCF-7 cells (13). IGFBP-1, inhibitor of IGF-1 signaling, decreases activation of IGF-1R and inhibits proliferation and survival in MCF-7 cells (14).

Tamoxifen, a selective estrogen receptor modulator and antagonist for estrogen receptor alpha (ERα) is a commonly prescribed adjuvant treatment for patients presenting with ERα-positive breast cancer. IGFBP-1 has also been shown to mediate the decrease in cell viability observed in tamoxifen-treated MCF-7 cells (15). In spite of the clinical benefit of tamoxifen treatment, about 40% of the patients develop resistance to tamoxifen over the course of treatment (16). It has been found that the loss of IGF-1R expression is one of most significant characteristics of acquired tamoxifen resistance (17). As a result, it was hypothesized that the accumulation of IGFBP-1 upon long-term tamoxifen treatment would result in the loss of IGF-1R expression, and eventually lead to the development of tamoxifen resistance.

In this study, initially we discovered that both tamoxifen resistant MCF-7 and T-47D cells expressed higher level of IGFBP-1 compared to parental cells. Then we found that both IGFBP-1 overexpressing MCF-7 and T-47D (MCF7-BP1 and T47D-BP1) cells shared some similarities with the corresponding TamR cells, such as the reduction of IGF-1R expression and increased Erk phosphorylation. Furthermore, we shown that both MCF7-BP1 and T47D-BP1 were tamoxifen-nonresponsive. Moreover, we found the transient knockdown of IGFBP-1 expression in these stable cells resulted in the reduced level of pErk and re-sensitized the cells to tamoxifen. Finally, we demonstrated the transient knockdown of IGFBP-1 restored the tamoxifen sensitivity in MCF7-TamR and T47D-TamR cells. Taken together, our data revealed a new mechanism of tamoxifen action that contributed to the development of tamoxifen resistance.

MATERIALS AND METHODS

Cell Culture

MCF-7 and T-47D breast cancer cells were purchased from ATCC (ATCC, Manassas, VA). All cells lines were maintained in maintenance DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). All cell lines for experiments were lower than passages 35 and both nucleotide and protein purifications were performed on cell lines at similar confluency.

Establishment of Stably Transfected Cells

Human IGFBP-1 expression vector (NM_000596) and the vector devoid of *IGFBP-1* ORF were purchased from OriGene (Rockville, MD). Plasmid transfection was performed using Lipofectamine 3000 reagent in serum-free Opti-MEM (Life Technologies, Carlsbad, CA) according to the manufacture's protocol. After 96 h of transfection, cells were washed with 1X PBS, and allowed to recover in maintenance media for 24 h then washed with 1X PBS followed by the addition of fresh maintenance media containing 800 or 400 μg/mL Geneticin (Life Technologies, Carlsbad, CA) for MCF-7 and T-47D cells, respectively. Untransfected cells were treated with Geneticin every 5 days until all cells were killed to demonstrate efficacy of Geneticin. All stably transfected cells were validated after selection by immunoblot and qRT-PCR. The stably transfected cell lines with IGFBP-1 containing plasmid were named MCF7-BP1 or T47D-BP1 and cells containing the vector devoid of *IGFBP-1* ORF were named MCF7-EV and T47D-EV.

Establishment of Tamoxifen Resistance (TamR) Cells

The method of establishing tamoxifen resistance cells was previously described (18). Briefly, cells were exposed 1 μM 4-hydroxytamoxifen (4-OHT) (Fluka, St. Louis, MO) in maintenance media. After 72 h of exposure, spent media was removed and new maintenance media containing 1 μM 4-OHT was added. After 21 days of 4-OHT exposure, cells that remained were allowed to recover and grow in fresh maintenance media. Cells were then split and maintained in maintenance media containing 1 μM 4-OHT. The cell lines generated by this method were named MCF7-TamR and T47D-TamR.

shRNA Knockdown

Human IGFBP-1 shRNA plasmid kit (Locus ID 3484) was purchased from OriGene (Rockville, MD). Plasmid transfection was performed using Lipofectamine 3000 reagent in serum-free Opti-MEM (Life Technologies, Carlsbad, CA) according to the manufacture's protocol. After 96 h of shRNA knockdown, cells

were harvested and the expression of IGFBP-1 was measured by immunoblot.

Cell Treatment

4-hydroxytamoxifen (4-OHT) (Sigma-Aldrich, St. Louis, MO) treatment was previously described (15). Briefly, 48 h prior to the treatment, cells were washed with 1X PBS and maintenance media was replaced with phenol red-free DMEM supplemented with 1% charcoal-stripped FBS (CS media) (Life Technologies, Carlsbad, CA). Cells were then washed with 1X PBS and treated with indicated concentrations of 4OHT in serum-free DMEM for 5 days. Ethanol was used to dissolve 4OHT. PD98059 (Life Technologies, Carlsbad, CA) was used to block the activation of MAP kinase (MEK). Forty eight hours prior to the treatment, cells were washed with 1X PBS and maintenance media was replaced with phenol red-free DMEM supplemented with 1% charcoal-stripped FBS (CS media) (Life Technologies, Carlsbad, CA). Cells were then washed with 1X PBS and treated with indicated concentrations of PD98059 in serum-free DMEM for 5 days. Ethanol was used to dissolve PD98059. For the EGF treatment, recombinant human EGF (Life Technologies, Carlsbad, CA) was used. Forty eight hours prior to the treatment, cells were washed with 1X PBS and maintenance media was replaced with phenol red-free DMEM supplemented with 1% charcoal-stripped FBS. After 24 h, cells were washed with 1X PBS and starved with serum and phenol red-free DMEM. After 24 h, cells were washed with 1X PBS and treated with serum and phenol red-free DMEM with the addition of indicated amount of EGF. Deionized water was used to dissolve the lyophilized EGF. Cells were harvested in 5 days.

Total RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies, Carlsbad CA) followed by on-column DNA digestion using Purelink DNase Set (Life Technologies, Carlsbad CA). cDNA was synthesized from 1 μg total RNA using the High Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad CA) and used as template in subsequent quantitative real-time PCR (RT-qPCR) reactions. qRT-PCR was performed using SYBR Green Master Mix (Life Technologies, Carlsbad CA) and the 7300 Real-Time PCR system (Bio-Rad, Hercules, CA). Primer pairs used for qRT-PCR: human IGFBP-1 forward 5′-CTA-TGA-TGG-CTC-GAA-GGC-TC-3′; reverse 5′-TTC-TTG-TTG-CAG-TTT-GGC-AG-3′ (19). Human IGF-1R forward 5'-GCA-CCA-TCT-TCA-AGG-GCA-ATT-TG-3′; reverse 5′-AGG-AAG-GAC-AAG-GAG-GAC-CAA-GG-3′. Human RPL30 gene was used as the internal control to normalize for mRNA in qRT-PCR reactions. Human RPL30 forward 5′-ACA-GCA-TGC-GGA-AAA-TAC-TAC-3′; reverse 5′-AAA-GGA-AAA-TTT-TGC-AGG-TTT-3′ (20).

Immunoblot Analysis

To prepare samples for immunoblot analysis, cells were harvested with RIPA lysis buffer containing protease and phosphatase inhibitor cocktails (Prod# 89901, 1862209, and 186249, Thermo Scientific, Rockford, IL). After lysis, cells were centrifuged at 12,000 × g for 15 min at 4°C, supernatant was collected protein concentrations was determined by BCA assay (Thermo Scientific, Rockford, IL). 30–75 μg total protein was resolved using Bolt 4–12% Bis-Tris Plus gels and transferred to PVDF membrane (Life Technologies, Carlsbad, CA). PVDF membranes were blocked in 1X Tris-buffered saline-0.1% Tween 20 (TBST) containing 5% fat-free milk at room temperature for 1 h with slow agitation. Membranes were then washed with 1X TBST three times and primary antibody was added and allowed to incubate overnight at 4°C. The following primary antibodies including dilution factor in 5% milk TBST were used in the current study: IGFBP-1 (#31025, Cell Signaling Technology, Danvers, MA); IGF-1R (#3027, Cell Signaling Technology Danvers, MA); P-IGF-1R (Tyr 1131) (#3021, Cell Signaling Technology, Danvers, MA); p44/42 MAPK (Erk1/2) (#9102, Cell Signaling Technology, Danvers, MA); P-p44/42 MAPK (T202/204) (#4377, Cell Signaling Technology, Danvers, MA); EGFR (#4267, Cell Signaling Technology, Danvers, MA); P-EGFR (Tyr 1068) (#3777, Cell Signaling Technology, Danvers, MA); Integrin β1 (sc-374429, Santa Cruz Biotechnology, Dallas, TX); β-actin (sc-47778, Santa Cruz Biotechnology, Dallas, TX). The dilution ratio for primary antibodies from Cell Signaling Technology was 1:1,000; The dilution ratio for primary antibodies from Santa Cruz Biotechnology was 1:2,000. After primary antibody incubation, membranes were washed three times with 1X TBST then incubated with anti-rabbit IgG conjugated to horseradish peroxidase (#7074, Cell Signaling Technology, Danvers, MA) or anti-mouse IgG conjugated to horseradish peroxidase (sc-81178, Santa Cruz Biotechnology, Dallas, TX) with dilution ratio of 1:5,000 at room temperature for 1 h. After washing membranes with 1X TBST three times, chemiluminescence reagent (34076, Thermo Scientific, Rockford, IL) was added and detected using Gel Doc™ XR ChemiDoc™ imaging system (BioRad, Hercules, CA) followed by quantification using ImageJ (NIH). Restore plus western blot buffer (46430, Thermo Scientific, Rockford, IL) was used to strip membranes of antibodies prior to probing for loading control where needed.

Extracellular IGFBP-1 Measurement

The method was previously described (15). Briefly, media was collected and concentrated with centrifugal filter units (UFC800396, MillporeSigma, Burlington, MA) at 4°C with the speed of 4,000 rpm for 1 h. Once centrifugated, media was collected with an addition of protease inhibitor cocktail (Prod #1862209, Thermo Scientific, Rockford, IL). Total protein concentration of concentrated media was measured by BCA assay, and the level of extracellular IGFBP-1 was determined by immunoblot analysis as previously described. For the external loading control, same amount of total protein (30 μg) of concentrated media samples were resolved by Bolt 4–12% Bis-Tris Plus gels. The gels were then washed with deionized water for 5 min and stained with Coomassie blue for 1 h. Thereafter, gels were destained with deionized water overnight. Gels were then imaged with FOTODYNE gel imager (FOTODYN INCORPORATED, Hartland, WI). The same intensity of protein band indicated the equivalent loading of samples.

Cell Viability Assay

After 5 days of treatments, cells were trypsinized and harvested with 1X PBS. The cell numbers were determined by counting via hemocytometer and compared to the vehicle-treated samples, which were normalized to 100%.

Statistical Analysis

All statistical analysis was performed by one-way ANOVA, Tukey's *post-hoc* test using Prism 6 (GraphPad, San Diego, CA). Differences were considered significant if $p \leq 0.05$ and the error bars are $\pm$ SEM.

RESULTS

MCF7-TamR and T47D-TamR Expressed More IGFBP-1 and the Establishment of MCF7-BP1 and T47D-BP1 Stable Cell Lines

Previously, insulin-like growth factor binding protein-1 (IGFBP-1) induction in 4-hydroxytamoxifen (4-OHT)-treated breast cancer cells was shown to mediate the efficacy of 4-OHT (15). To determine if IGFBP-1 is critical for the development of tamoxifen resistance in breast cancer cells, the level of IGFBP-1 in MCF-7 parental cells (MCF7-P) and MCF-7 tamoxifen resistant cell (MCF7-TamR), as well as in T-47D parental (T47D-P) and T47D-TamR was determined. Both MCF7-TamR and T47D-TamR expressed higher levels of IGFBP-1 compared to parental cells that did not have detectable levels of IGFBP-1 (**Figure 1A**). These data suggested that IGFBP-1 may promote the development of tamoxifen resistance. To determine if IGFBP-1 exposure is sufficient for the development of tamoxifen resistance in breast cancer cells, MCF-7 and T-47D cells with stable overexpression of IGFBP-1 were generated. After selection, both extracellular and intracellular levels of IGFBP-1 were determined by immunoblot. For both MCF-7 and T-47D cells, the level of intracellular and extracellular IGFBP-1 in empty vector (EV) controls was low and similar to the parental cell lines. For MCF-7 and T-47D cells stably expressing the IGFBP-1 expression vector (designated BP1 for each cell line), high levels of both intracellular and

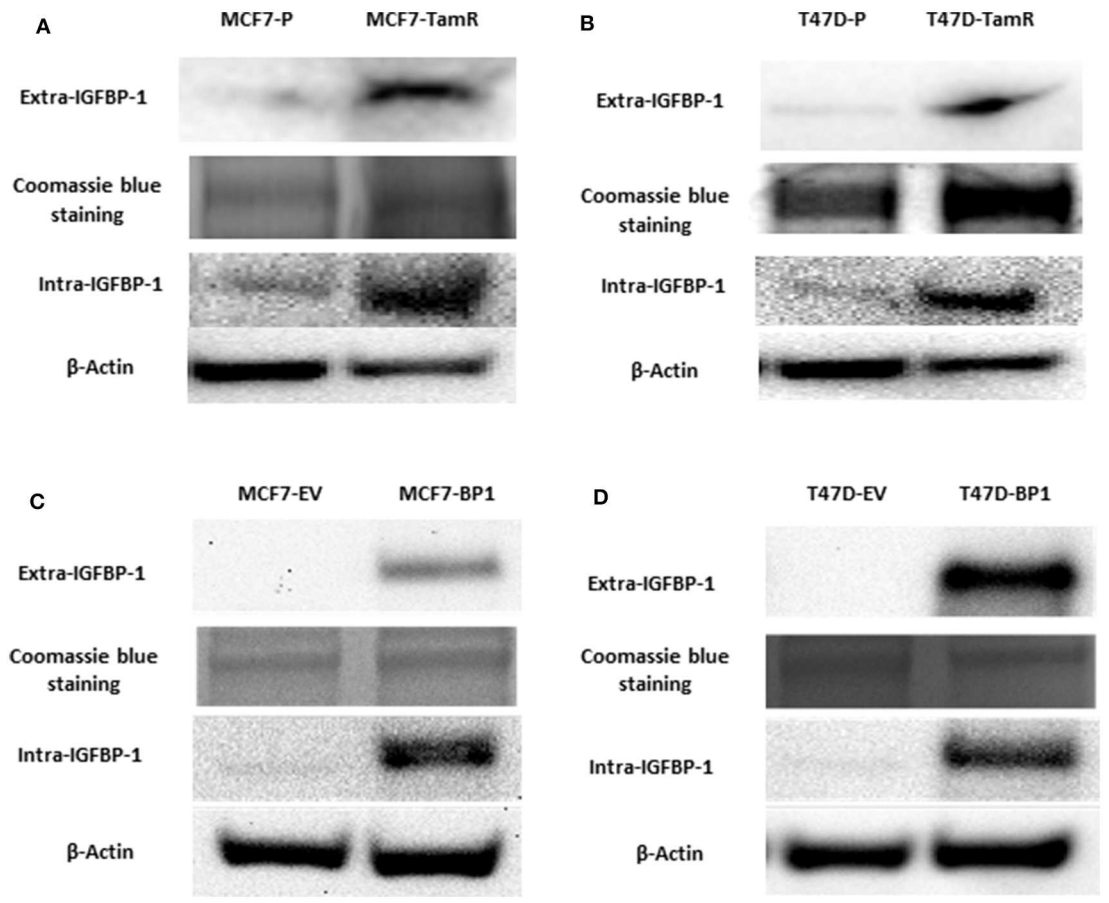

FIGURE 1 | MCF7-TamR and T47D-TamR expressed more IGFBP-1 and the establishment of MCF7-BP1 and T47D-BP1 stable cell lines. Immunoblot analysis of IGFBP-1 expression in MCF-7 and T-47D stable cells. **(A)** Measurement of IGFBP-1 expression in MCF7-P and MCF7-TamR (left), and in T47D-P and T47D-TamR (right); **(B)** measurement of IGFBP-1 expression in MCF7-EV and MCF7-BP1 (left), and in T47D-EV and T47D-BP1 (right). The Coomassie blue staining indicated the even loading of the proteins from the concentrated media. Results are the representatives of 3 independent experiments. Extra-IGFBP-1: extracellular IGFBP-1; intra-IGFBP-1: intracellular IGFBP-1.

extracellular IGFBP-1 were observed compared to the EV controls (**Figure 1B**). Additionally, MCF7-BP1 and T47D-BP1 cells had a significant induction of IGFBP-1 transcript compared to EV controls (data not shown).

Expression of IGF-1R Decreased in MCF7-BP1 and T47-BP1 Cells

It has been reported that the acquired tamoxifen resistance in MCF-7 and T-47D cells is associated with the decreased IGF-1R transcription and expression (17, 21). In agreement with these reports, the TamR cells generated (**Figure 1**) expressed significantly less IGF-IR compared to the parental cells. Since IGF-1R expression is associated with tamoxifen resistance, IGF-1R expression was used as an indicator of tamoxifen resistance MCF7-BP1 and T47D-BP1 cells. In both cell lines, IGF-1R expression was decreased compared to MCF7-EV and T47D-EV cells (**Figure 2A**). Additionally, low levels of IGF-1R transcript were observed in the each TamR cells line when compared to

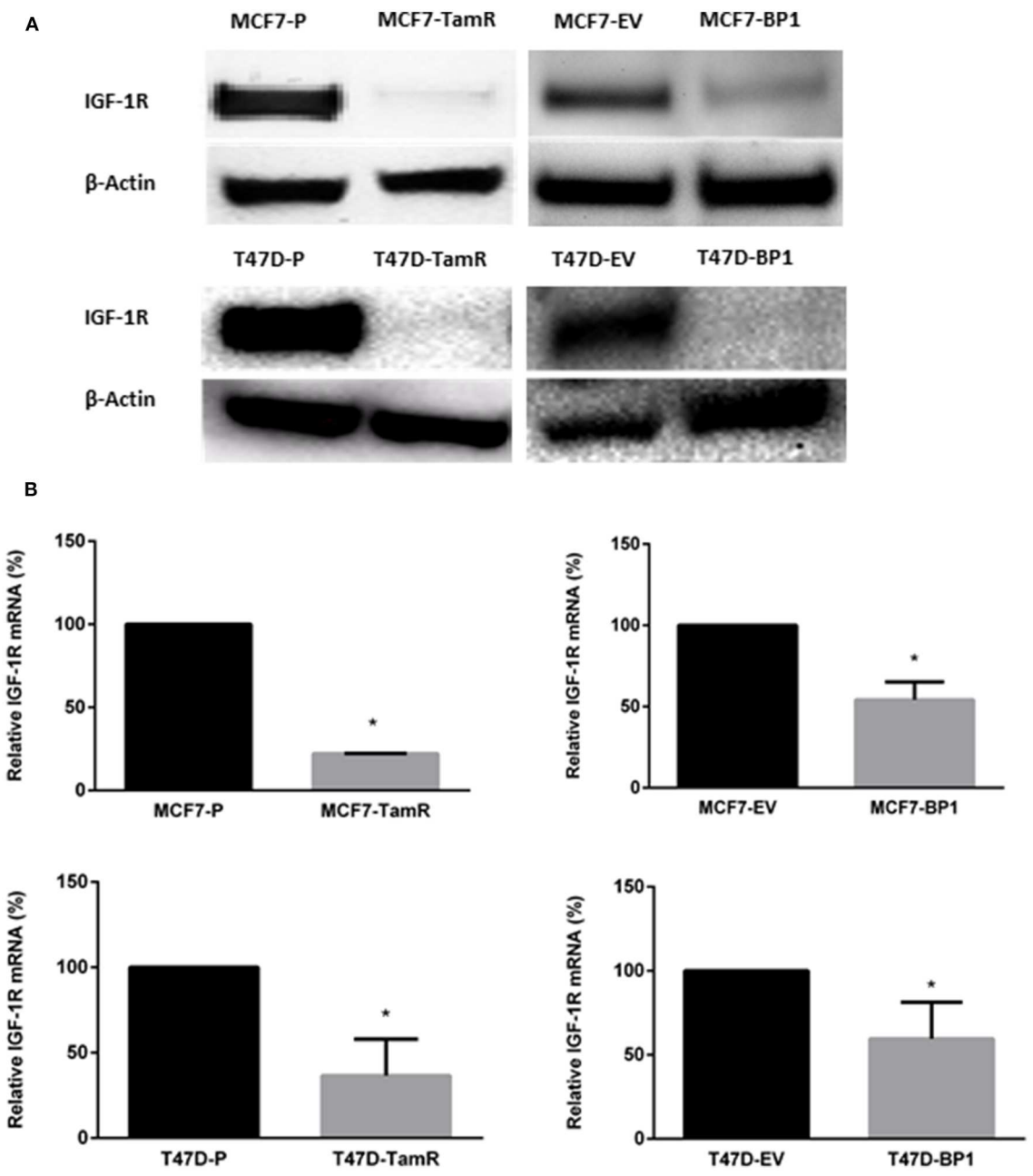

FIGURE 2 | Expression of IGF-1R decreased in MCF7-BP1 and T47-BP1 cells. **(A)** Immunoblot analysis of IGF-1R protein expressions in MCF-7 and T-47D cells. **(B)** qRT-PCR analysis of IGF-1R mRNA levels in MCF-7 and T-47D cells. Results are the average of 3 independent experiments, and error bars are the standard error of the mean. *$p < 0.05$.

parental cells consistent with previous reports (17). Similar to the observations of decreased IGF-1R expression in TamR breast cancer cells, IGF-1R expression was decreased in MCF7-BP1 and T47D-BP1 compared to MCF7-EV and T47D-EV (**Figure 2B**). These results suggested that sustained exposure to IGFBP-1 in breast cancer cells contributes to the development of tamoxifen resistance by altering the IGF-1 signaling pathway.

Sustained IGFBP-1 Exposure Increases EGFR Signaling

The upregulation of epidermal growth factor receptor (EGFR) is commonly observed in tamoxifen resistance MCF-7 cells (22–25). In addition, the increase of EGFR phosphorylation at tyrosine 1068 has been reported (26). Also, it has been shown that the phosphorylation of Erk is elevated in MCF7-TamR cells (21, 23, 24). In the TamR breast cancer cells developed for this study, alterations in signaling pathways consistent with previous reports were observed (**Figure 3A**). Additionally, MCF7-BP1 cells expressed higher level of phospho-EGFR, EGFR, and phospho-Erk compared to MCF7-EV (**Figure 3A**). Given that the expression of EGFR was upregulated in MCF7-TamR and MCF7-BP1, stimulation of cells by EGFR was determined. While the viability of MCF7-P and MCF7-EV was not significantly increased upon EGF treatment, the viability of both MCF7-TamR and MCF7-BP1 was increased by EGF in a dosage-dependent manner (**Figure 3B**). Additionally, an upregulation of EGFR expression in T47D-TamR compared to T47D-P was observed and phospho-EGFR as well as phospho-Erk observed (**Figure 3C**). Similar to the T47D-TamR, the levels of EGFR, phospho-EGFR, and phospho-Erk were increased in T47D-BP1 compared to T47D-EV (**Figure 3C**). Similar to the observations with the MCF-7 and derived cells lines, EGF did not increase the viability of both T47D-P and T47D-EV while EGF treatment increased the viability of both T47D-TamR and T47D-BP1 (**Figure 3D**). Taken together, these data indicated that the sustained exposure to IGFBP-1 results in increased EGFR signaling in breast cancer cells and this transition to EGF sensitivity is similar to the transition that occurs during development of tamoxifen resistance in breast cancer cells.

Sustained IGFBP-1 Exposure Results in the Development of Tamoxifen Resistance in Breast Cancer Cells

EGFR pathway is the predominant pathway related to tamoxifen resistance (27). In particular, it has been suggested that elevated expression of EGFR may serve as an indication of anti-estrogen

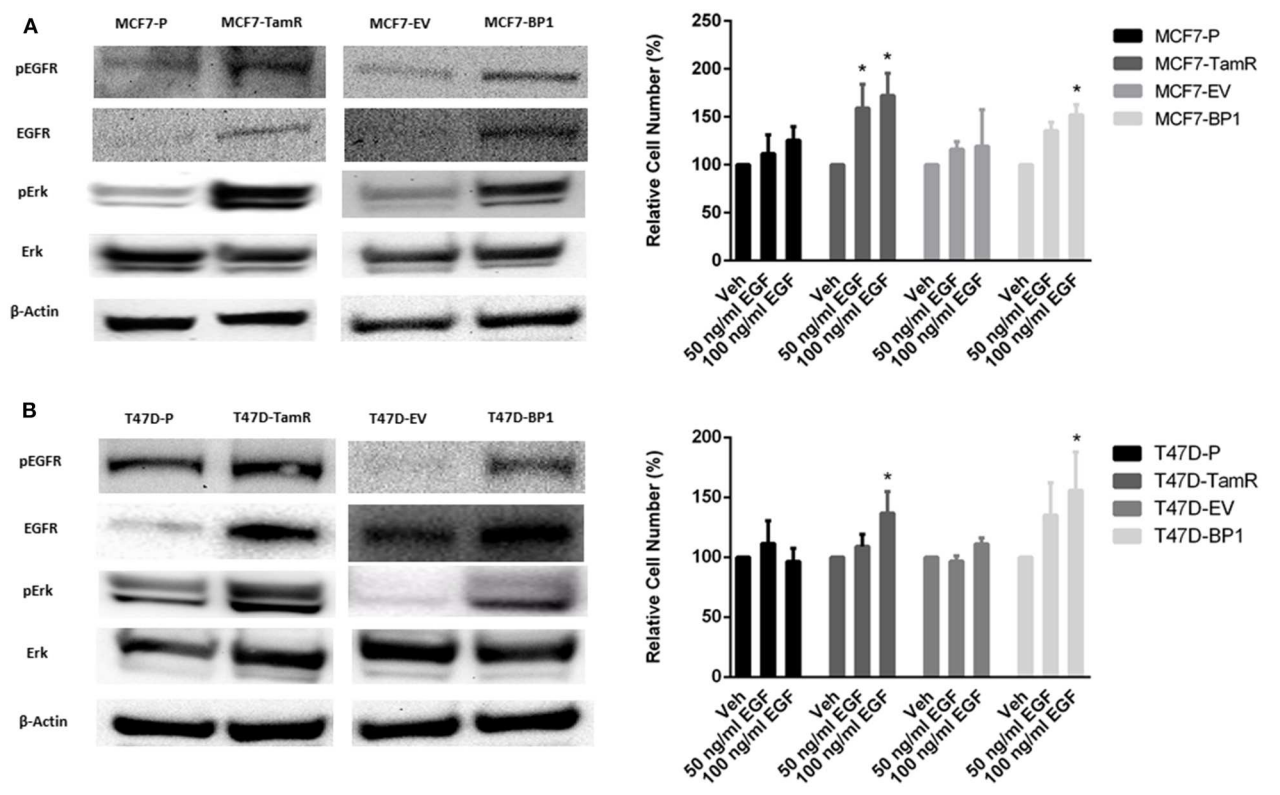

FIGURE 3 | Sustained IGFBP-1 exposure increases EGFR signaling. **(A)** Immunoblot analysis of phospho-EGFR (Y1068), EGFR, phospho-Erk, and Erk in MCF-7 cells (top); measurement of relative cell number (%) in MCF-7 cells after 5 days of EGF treatment (bottom). **(B)** Immunoblot analysis of phospho-EGFR (Y1068), EGFR, phospho-Erk, and Erk in T-47D cells (top); measurement of relative cell number (%) in T-47D cells after 5 days of EGF treatment (bottom). Results are the average of 3 independent experiments, and error bars are the standard error of the mean. *$p < 0.05$.

resistance in ER α positive breast cancer cells (28–30). Given that both of the MCF7-BP1 and T47D-BP1 had higher levels of EGFR, phospho-EGFR, and phospho-Erk, it was hypothesized that the long-term exposure to IGFBP-1 was sufficient for the development of tamoxifen resistance in breast cancer cells. MCF7-BP1 and T47D-BP1 cells were treated with 4-OHT, and cell numbers were measured after 5 days of treatment. While the viability of MCF7-P and MCF7-EV was reduced by the 4-OHT treatment in a dosage-dependent manner, the viability of MCF7-TamR was increased by the 4-OHT treatment, and the viability of MCF7-BP1 was not decreased by 4-OHT (**Figure 4**). These observations are consistent with previous reports reference previously in this contribution. To determine if the BP-1 variants of the MCF-7 and T-47D cells had a similar resistance to 4-OHT treatment, these cells were treated with 4-OHT and viability was determined by cell counts. Treatment with 4-OHT significantly decreased viability of T47D-P and T47D-EV cells, while cell viability was not significantly altered upon 4-OHT treatment T47D-TamR or T47D-BP1 cells. These data suggest that sustained exposure to IGFBP-1 is sufficient for the development of tamoxifen resistance in breast cancer cells.

MAPK Inhibition Reverses Tamoxifen Resistance in Breast Cancer Cells

Activation of Erk plays an important role in the development of tamoxifen resistance in ER α positive breast cancer cells (31–33). Previously, it was reported that MAPK inhibitor PD98059 inhibits proliferation in MCF-7 cells (34), while it had no significant effect in tamoxifen resistant MCF-7 cells (35). However, it was shown that the combination of 4-OHT and PD98059 decreased cell viability tamoxifen resistant MCF-7 cells. To determine if the IGFBP-1 expressing MCF-7 and T-47D cells were sensitive to PD98059 treatment or co-treatment with 4-OHT with PD98059, cell were treated, and viability was determined by cell count. In **Figure 5A**, the level of phospho-Erk was reduced by PD98059 and the co-treatment of tamoxifen and PD98059 in MCF7-EV. Consistently, the cell numbers of MCF7-EV were significantly decreased by the treatment of 4-OHT and PD98059, and the co-treatment of 4-OHT with PD98050 appeared to have an additive effect on killing MCF7-EV cells (**Figure 5A**). Both treatment with PD98059 or the co-treatment of 4-OHT with PD98059 effectively reduced the level of phospho-Erk in MCF7-BP1. Interestingly, while neither 4-OHT or PD98050 alone was able to reduce the cell numbers of MCF7-BP1 significantly, the co-treatment of both drugs decreased the viability of MCF7-BP1 cells (**Figure 5B**). Similar to MCF7-EV, single treatment of 4-OHT or PD98059 significantly reduced the cell numbers of T47D-EV, and the co-treatment of both drugs had an decreased T47D-EV cells (**Figure 5C**). In T47D-BP1 cells, viability was not reduced by either 4-OHT or PD98059, however co-treatment significantly reduced the viability of T47D-BP1cells (**Figure 5D**). Taken together, our data revealed that the activation of Erk in MCF7-BP1 and T47D-BP1 cells played a protective role against the 4-OHT treatment, and the inhibition of Erk activation by PD98059 re-sensitized the cells to 4-OHT suggesting that Erk activation in MCF7-BP1 and T47D-BP1 cells was a key element for tamoxifen resistance.

Knockdown of IGFBP-1 in MCF7-BP1 and T47D-BP1 Reduced the Level of Phospho-Erk and Sensitized the Cells to 4-OHT

Besides functioning to regulate IGF-1 action, IGFBP-1 is reported to be a stimulator of Erk in several cell types (36–38). A similar role for IGFBP-1 in breast cancer cells has not been reported. To determine if IGFBP-1 exposure results in the activation of Erk in breast cancer cells, IGFBP-1 was transiently reduced in MCF7-BP1 and T47D-BP1 cells and Erk phosphorylation was measured. The transient knockdown of IGFBP-1 for 96 h effectively reduced both extracellular and intracellular IGFBP-1 accumulation and this knockdown also reduced the accumulation of phospho-Erk in both MCF7-BP1 and T47D-BP1 cells (**Figures 6A,B**). The previous experiments demonstrated that inhibition of phosphor-Erk accumulation sensitized both MCF7-BP1 and T47D-BP1 cells to 4-OHT treatment (**Figure 5B**), it was reasoned that knockdown of IGFBP-1 would similarly sensitize MCF7-BP1 and T47D-BP1 cells to 4-OHT treatment. Knockdown of IGFBP-1 in MCF7-BP1 resulted in a result in a significant decrease in viability

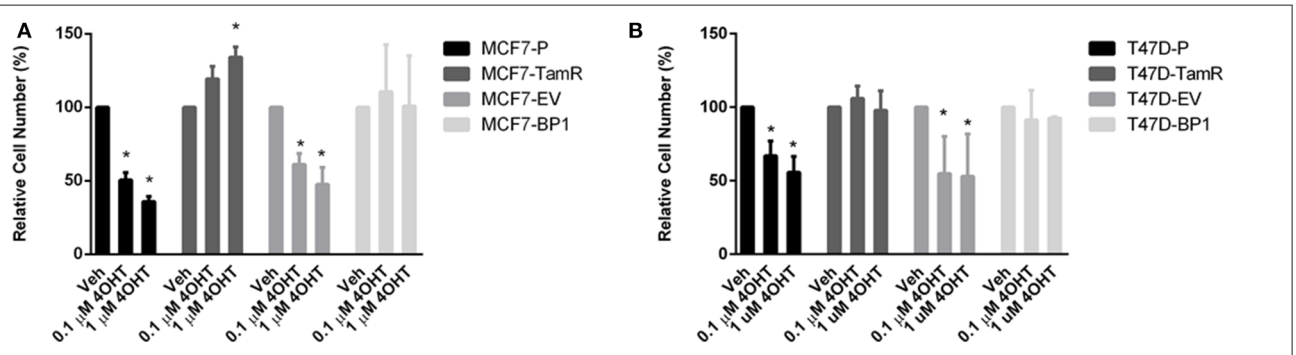

FIGURE 4 | Sustained IGFBP-1 exposure results in the development of tamoxifen resistance in breast cancer cells. **(A)** Measurement of relative cell number (%) in four different MCF-7 cell lines under 4-hydroxytamoxifen (4OHT) treatment for 5 days. **(B)** Measurement of relative cell number (%) in four different T-47D cell lines under 4-hydroxytamoxifen (4OHT) treatment for 5 days. Results are the average of 3 independent experiments, and error bars are the standard error of the mean. *$p < 0.05$.

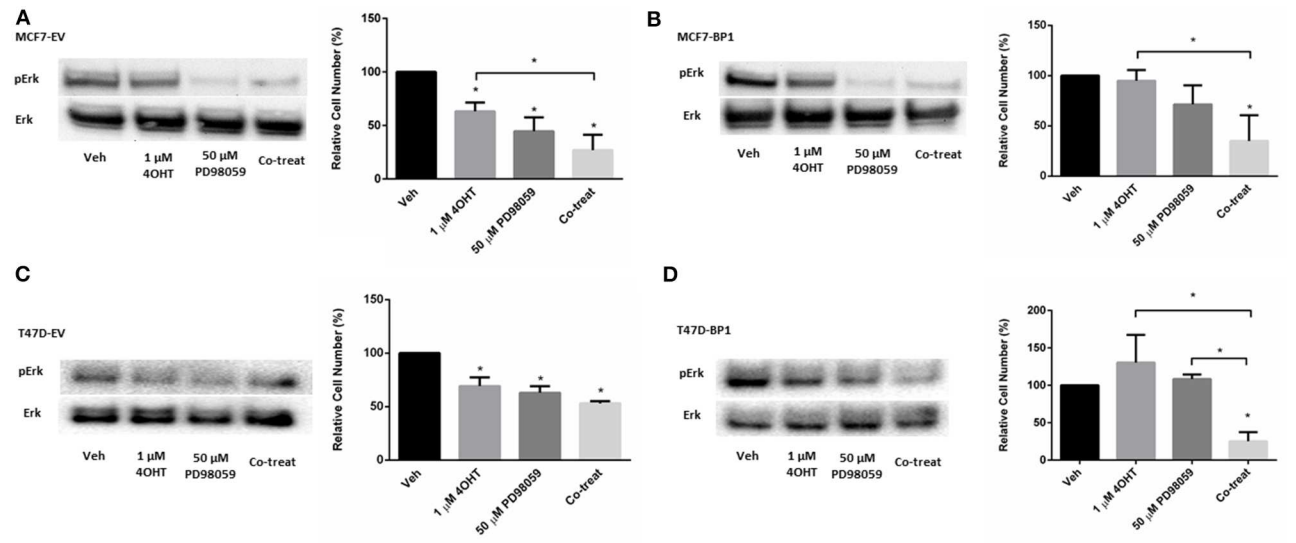

FIGURE 5 | MAPK inhibition reverses tamoxifen resistance in breast cancer cells. **(A)** Left: immunoblot analysis of phospho-Erk (pErk) of treated MCF7-EV; right: measurement of relative cell number (%) of MCF7-EV under different treatments for 5 days. **(B)** Left: immunoblot analysis of phospho-Erk (pErk) of treated MCF7-BP1; right: measurement of relative cell number (%) of MCF7-BP1 under different treatments for 5 days. **(C)** Left: immunoblot analysis of phospho-Erk (pErk) of treated T47D-EV; right: measurement of relative cell number (%) of T47D-EV under different treatments for 5 days. **(D)** Left: immunoblot analysis of phospho-Erk (pErk) of treated T47D-BP1; right: measurement of relative cell number (%) of T47D-BP1 under different treatments for 5 days. Results are the average of 3 independent experiments, and error bars are the standard error of the mean. *$p < 0.05$.

when treated with 1 μM 4-OHT compared to non-targeting control. Similar to MCF7-BP1 cells, neither the treatment of 4-OHT nor the knockdown of IGFBP-1 decreased the cell numbers of T47D-BP1, whereas the combination of 4-OHT treatment with IGFBP-1 knockdown significantly reduced the cell numbers (**Figure 6B**). These data suggest that exposure to IGFBP-1 is involved in the development of tamoxifen resistance in breast cancer cells. Furthermore, these data suggest that elevated IGFBP-1 levels stimulate Erk activation and resulting in tamoxifen resistance in breast cancer cells.

Transient Knockdown of IGFBP-1 Restores Tamoxifen Sensitivity in Breast Cancer Cells

To determine if elevated IGFBP-1 expression is required for tamoxifen sensitivity in breast cancer cells, transient knockdown of IGFBP-1 expression was performed in both MCF7-TamR and T47D-TamR cells. Transient knockdown of IGFBP-1 for 96 h in MCF7-TamR sufficiently reduced the accumulation of IGFBP-1 and phospho-Erk (**Figure 7**). Transient knockdown of IGFBP-1 reduced cell viability suggesting that IGFBP-1 is a prosurvival factor in MCF-TamR cells. Furthermore, transient knockdown of IGFBP-1 in MCF7-TamR cells restored tamoxifen sensitivity as indicated by decreased cell numbers upon 4-OHT treatment preceded by IGFBP-1 knockdown. Similar experiments were performed in T47D-TamR, however, IGFBP-1 knockdown was less robust in this cell line. In the T47D-TamR phospho-Erk accumulation was not reduced upon IGFBP-1 knockdown while the knockdown did significantly reduced cell viability of T47D-TamR. These data support the conclusion IGFBP-1 is a pro-survival signal in TamR breast cancer cells. When T47D-TamR were treated with 1 μM 4-OHT after IGFBP-1 knockdown, a reduction of viability was observed, however this reduction was not statistically significant ($p = 0.0950$) like what was observed in the MCF-7 model. Taken together, these results provide evidence that IGFBP-1 contributes to the development of tamoxifen resistance in breast cancer cells and is a pro-survival signal for tamoxifen resistant breast cancer cells.

DISCUSSION

Tamoxifen resistance remains a clinically relevant complication for women receiving adjuvant breast cancer treatment. Much work has been directed toward understanding tamoxifen resistance and several mechanisms or chemoresistance have been proposed. These range from the loss or alteration of ER α expression to the activation of alternative growth factor pathways observed in tamoxifen resistant cells (39–41). The data described in this contribution provides a link between the G protein-coupled estrogen receptor 1 (GPER1)-mediated IGFBP-1 accumulation associated with tamoxifen treatment in breast cancer cells (15) with the alteration in growth factor signaling previously reported (17). Furthermore, these data demonstrate that sustained IGFBP-1 exposure results in tamoxifen resistance and IGFBP-1 expression is a critical component of chemoresistance in breast cancer cells. Taken together, these data provide support for the conclusion that IGFBP-1 is sufficient

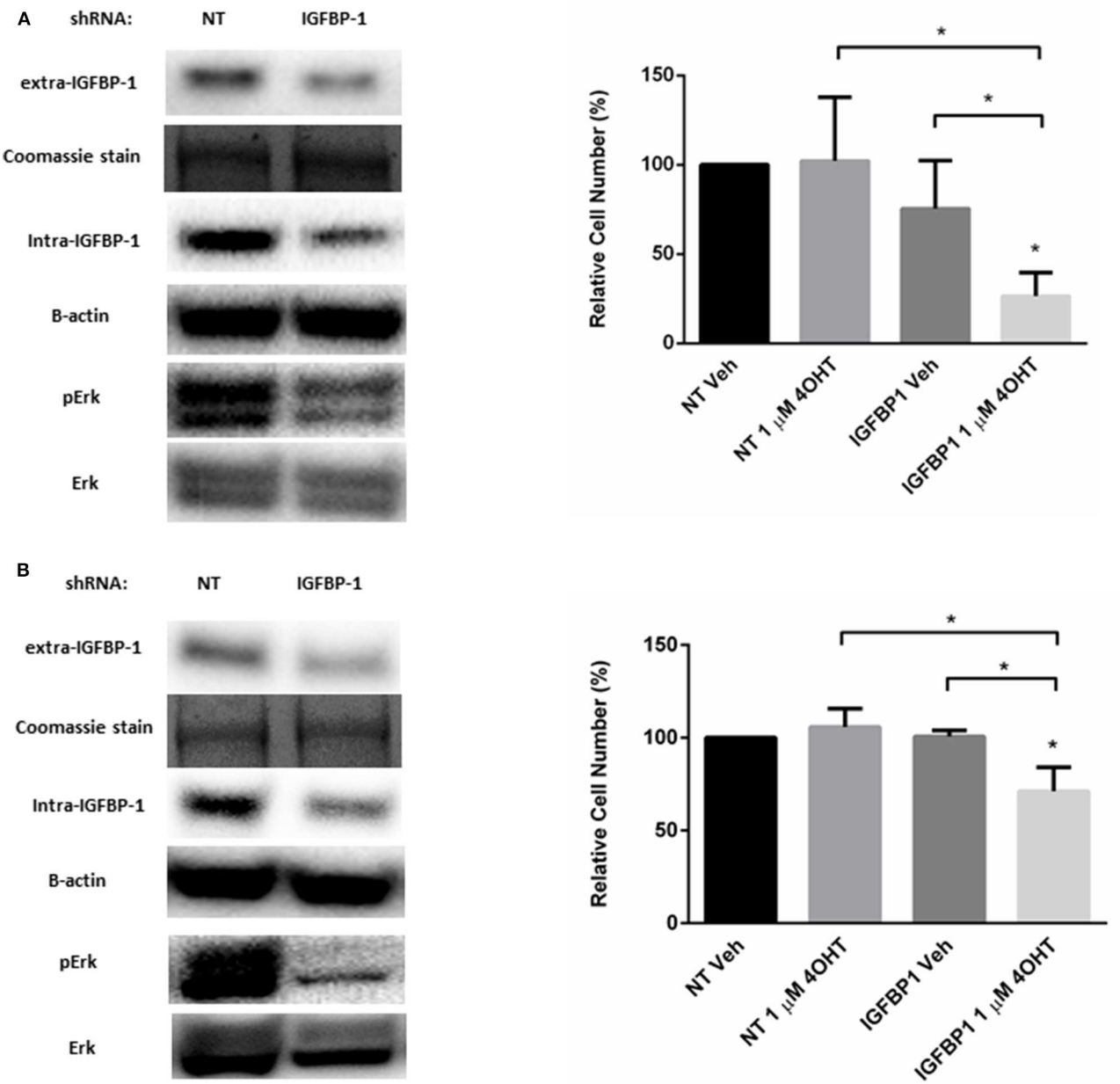

FIGURE 6 | Knockdown of IGFBP-1 in MCF7-BP1 and T47D-BP1 reduced the level of phospho-Erk and sensitized the cells to 4-OHT. **(A)** Immunoblot analysis of IGFBP-1, phospho-Erk, and Erk after transfected with non-targeting (NT) shRNA or IGFBP-1 shRNA in MCF7-BP1 (left); measurement of relative cell number (%) in MCF7-BP1 after transfected with NT shRNA or IGFBP-1 shRNA and treated with either vehicle or 1 μM 4-OHT (right). **(B)** Immunoblot analysis of IGFBP-1, phospho-Erk, and Erk after transfected with non-targeting (NT) shRNA or shRNA for IGFBP-1 in T47D-BP1 (left); measurement of relative cell number (%) in T47D-BP1 after transfected with NT shRNA or IGFBP-1 shRNA and treated with either vehicle or 1 μM 4-OHT (right). Results are the average of 3 independent experiments, and error bars are the standard error of the mean. *$p < 0.05$.

to confer tamoxifen resistance in breast cancer cells and is a prosurvival factor for chemoresistant breast cancer cells.

IGFBPs are known to have many functions in cells and these can be intracellular and/or extracellular. Complete elucidation of the role that IGFBP-1 plays in tamoxifen resistance will require continued discovery and analysis of IGFBP-1-mediated cellular pathways. IGFBP-1 is anti-proliferative for MCF-7 cells (14) and T-47D cells (42) was demonstrated to inhibit the mobility of human metastatic breast cancer cell line MDA-231BO (43). Data from these reports supports a tumor suppressive role for IGFBP-1 in breast cancer cells. However, in the tamoxifen resistant breast cancer cell the role for IGFBP-1 has been altered. The results reported herein suggest that IGFBP-1 has a prosurvival role in the

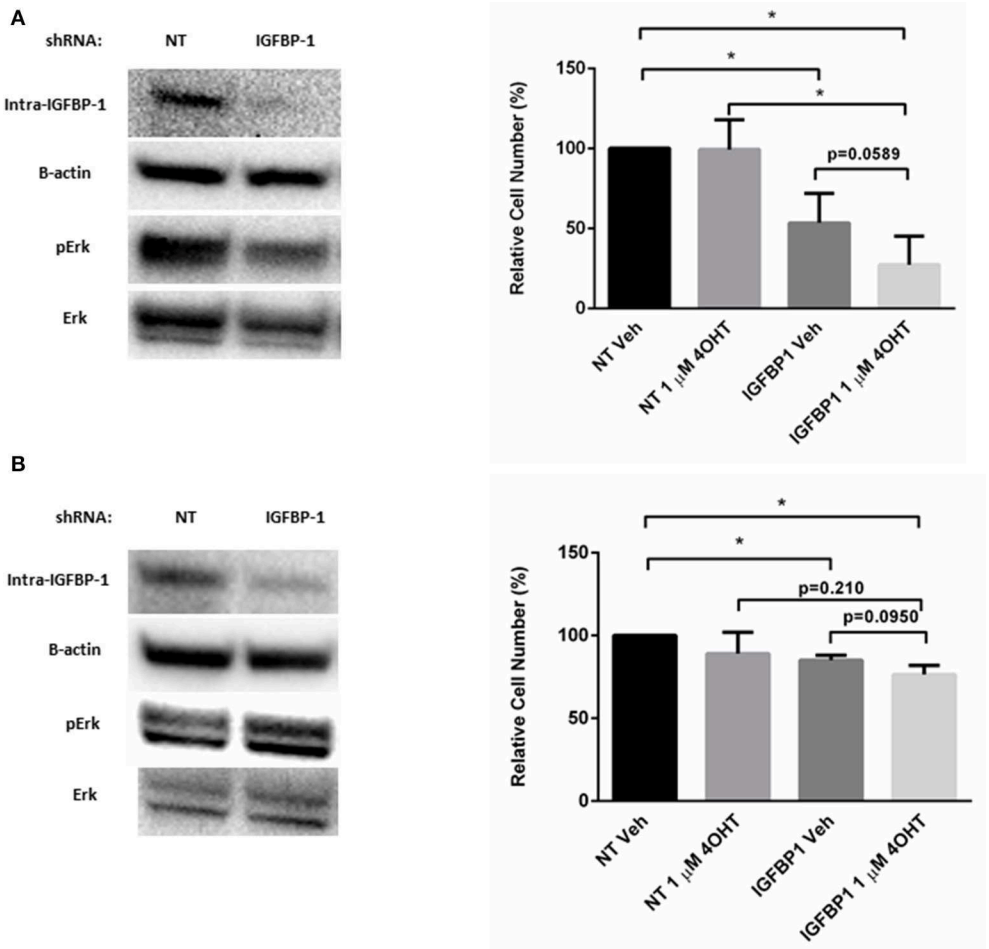

FIGURE 7 | Knockdown of IGFBP-1 restores 4-OHT sensitivity in breast cancer cells. **(A)** Immunoblot analysis of IGFBP-1, phospho-Erk, and Erk after transfected with non-targeting (NT) shRNA or IGFBP-1 shRNA in MCF7-TamR (left); measurement of relative cell number (%) in MCF7-TamR after transfected with NT shRNA or IGFBP-1 shRNA and treated with either vehicle or 1 μM 4-OHT (right). **(B)** Immunoblot analysis of IGFBP-1, phospho-Erk, and Erk after transfected with non-targeting (NT) shRNA or shRNA for IGFBP-1 in T47D-TamR (left); measurement of relative cell number (%) in T47D-TamR after transfected with NT shRNA or IGFBP-1 shRNA and treated with either vehicle or 1 μM 4-OHT (right). Results are the average of 3 independent experiments, and error bars are the standard error of the mean. *$p <$ 0.05.

tamoxifen resistant breast cancer cell and that sustained IGFBP-1 exposure is sufficient for the development tamoxifen resistance. Thus far, the mechanism by which IGFBP-1 acts to enhance cell viability in breast cancer cells has not been determined. One possible mechanism that underlies the prosurvivla role for IGFBP-1 in breast cancer cells is the known interaction with integrin α5β1. The integrin recognition sequence Arg-Gly-Asp (RGD) of IGFBP-1 interacts with integrin α5β1 resulting in the activation of Erk in multiple cell lines (36–38). Integrin α5β1 has been implicated in solid tumors, and it was shown to promote the adhesion and invasion for breast cancer cells (44).

In addition to the role that IGFBP-1 has in the activation of cellular pathways, the regulation of IGFBP-1 expression and activity will need to be investigated to include analyzing the phosphorylation status of IGFBP-1 in the tamoxifen resistant breast cancer cell context. There are three major sites of phosphorylation in the linker domain of human IGFBP-1, which are Ser 98, Ser 101, and Ser 119 (45). The phosphorylation on these residues contributes to increased binding affinity of IGFBP-1 to IGF-1 (46). Non-phosphorylated IGFBP-1 has lower IGF-1 binding affinity and thus potentiates IGF-1R activation (8) which was also demonstrated to activate Erk (47, 48). One explanation for the data presented here is that IGFBP-1 is expressed but not phosphorylated and therefore potentiates Erk activation in tamoxifen resistant breast cancer cells. This line of investigation will require analysis of the kinase involved in IGFBP-1 phosphorylation such as CK1 and CK2 (49).

Differentiating the extracellular and intracellular roles of IGFBP-1 is necessary to fully understand the contribution of IGFBP-1 in breast cancer cells both during tamoxifen treatment and in the tamoxifen resistant state. For example, IGFBP-3, the most widely studied IGFBP family member, can be internalized and relocated to nucleus via its binding to transferrin and caveolin 1 (50). Once located in the nucleus, IGFBP-3 was shown to interact with histone-DNA complex and act as a transcriptional regulator for certain genes (51). Because

IGFBP-1 is expressed both intracellularly and extracellularly and shares high degree of homology of transferrin and caveolin 1 binding regions with IGFBP-3 (50). Until know, the only functions associated with IGFBP-1 in tamoxifen treated breast cancer cells have focused on the extracellular role of this protein. The potential involvement of intracellular IGFBP-1 and the contribution to tamoxifen resistance will need to be studied.

Taken together, these data reveal a novel role for IGFBP-1 in the development of tamoxifen resistance in breast cancer cells. In tamoxifen-sensitive cells, IGFBP-1 accumulation function to decrease cell viability while the long-term exposure to IGFBP-1 results in tamoxifen resistance. This suggests that IGFBP-1 is sufficient for the development of tamoxifen resistance. Furthermore, tamoxifen resistant cells have increased IGFBP-1 accumulation the viability of the cells is decreased when IGFBP-1 is reduced. This suggests that IGFBP-1 is a key prosurvival factor in the tamoxifen resistant cell state. These results may have clinical implications due to the possibility of monitoring IGFBP-1 expression as a marker of tamoxifen resistance.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

YZ contributed to the conception, the acquisition of data, analysis, and interpretation of the work as well as drafting the work and revision of the manuscript. JS contributed to data acquisition and revision of the manuscript. KH contributed to the conception and interpretation of the work, critical revision of the manuscript, final approval, and agrees to be accountable for all aspects of the work.

FUNDING

This work was supported by 5SC1GM127175 and 5T34GM007667 from the National Institute of General Medical Sciences of the National Institutes of Health.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 23 June 2020
doi: 10.3389/fendo.2020.00403

Mini Review: Opposing Pathologies in Cancer and Alzheimer's Disease: Does the PI3K/Akt Pathway Provide Clues?

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This minireview is a brief overview examining the roles of insulin-like growth factors (IGFs) and the PI3K/Akt pathway in two apparently unconnected diseases: Alzheimer's dementia and cancer. For both, increased age is a major risk factor, and, in accord with the global rise in average life expectancy, their prevalence is also increasing. Cancer, however, involves excessive cell proliferation and metastasis, whereas Alzheimer's disease (AD) involves cell death and tissue destruction. The apparent "inverse" nature of these disease states is examined here, but also some important commonalities in terms of the PI3K/Akt pathway, glucose utilization and cell deregulation/death. The focus here is on four key molecules associated with this pathway; notably, the insulin receptor substrate 1 (IRS-1), cellular tumor antigen p53 (p53), peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1) and low-density lipoprotein receptor–related protein-1 (LRP1), all previously identified as potential therapeutic targets for both diseases. The insulin-resistant state, commonly reported in AD brain, results in neuronal glucose deprivation, due to a dampening down of the PI3K/Akt pathway, including overactivity of the mammalian target of rapamycin 1 (mTORC1) complex, hyperphosphorylation of p53 and neuronal death. This contrasts with cancer, where there is overstimulation of the PI3K/Akt pathway and the suppression of mTORC1 and p53, enabling abundant energy and unrestrained cell proliferation. Although these disease states appear to be diametrically opposed, the same key molecules are controlling pathology and, with differential targeting of therapeutics, may yet provide a beneficial outcome for both.

Keywords: cancer, Alzheimer's, PI3K/Akt pathway, IGF-1, insulin, LRP1, PIN1, p53

OPEN ACCESS

Edited by:
Marco Falasca,
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Reviewed by:
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *06 April 2020*
Accepted: *20 May 2020*
Published: *23 June 2020*

Citation:
Barker RM, Holly JMP, Biernacka KM, Allen-Birt SJ and Perks CM (2020) Mini Review: Opposing Pathologies in Cancer and Alzheimer's Disease: Does the PI3K/Akt Pathway Provide Clues? Front. Endocrinol. 11:403. doi: 10.3389/fendo.2020.00403

BACKGROUND

In 2018 there were 17 million new cases of cancer and 9.6 million deaths worldwide (1). One of its most common forms is breast cancer, a leading cause of cancer mortality worldwide (2), with over two million new cases in 2018. Dementia is also a major cause of suffering and death globally, with 9.9 million new cases estimated each year (3); 60–70% of these are diagnosed as Alzheimer's disease (AD) (4). AD and breast cancer, as examples of each disease spectrum, are contrasted here

with respect to differences in the PI3K/Akt pathway. By comparing four specific key molecules, we hope to provide some insight into potential, differential therapeutic targeting. Although, due to the limitations of a mini-review we needed to narrow our selection, we acknowledge that additional molecules contributing to the inverse nature of these pathologies have also been reviewed previously (5).

Every normal cell in the body will acquire mutations over a lifetime, which may result in cancer. It has been clear for many years that the initiating mutations and neoplastic transformation may occur decades before symptoms become present and the cancer is diagnosed**.** Most breast cancers are epithelial tumors that develop from cells lining ducts or lobules: carcinoma *in situ,* and are located exclusively in the breast, tending to be detected by routine physical examination or mammography. Invasive breast cancer can spread however, to most organs, with the main sites being the lungs, liver, bone and brain. There are five main subtypes of breast cancer, depending on the expression of the estrogen, progesterone and human epidermal growth factor receptor 2 (HER2) receptors which dictate treatment strategies (6). One mutational profile often observed in many cancers is hyperactivity of the PI3K/Akt signaling pathway leading to deregulated control of cell proliferation (7). Another common feature associated with cancer risk and progression is chronic inflammation, which can be initiated by triggers, such as infections, obesity and autoimmune diseases, the effects of which can be mediated by cytokines, such as tissue necrosis factor (TNF) and interleukins (IL-1 and 6) (8).

As for cancer, the diagnosis of AD usually occurs long after the onset of neuropathology, often 10–20 years later, mainly because symptoms do not generally become evident until the brain has been severely compromised. Loss of short-term memory is usually the first symptom; later, cognitive failure and confusion, and finally an inability to carry out tasks required for successful daily living. Its two defining brain pathologies are the presence of amyloid plaques, comprised mainly of the toxic peptide Aβ42 (processed from the amyloid precursor protein (APP), which quickly fibrillises and deposits in the parenchyma of the brain, and hyperphosphorylated tau, which accumulates within neurones into neurofibrillary tangles (NFT). The parallel spread of these two pathologies across the brain, occurs over a long period before clinical symptoms become evident. Until recently, this has made early diagnosis and assessment of treatment effectiveness difficult. Positron emission tomography (PET) scans with ligands which register amyloid and NFT, as well as markers of neuroinflammation, are now available, helping diagnosis, clinical trial investigation and basic scientific discovery (9). Recent investigations with PET ligands in living patients suggest that symptoms are noticeable when amyloid and NFT both reach sufficiently high levels (10). The brain, separated from the peripheral immune system by the blood-brain-barrier (BBB), relies on its innate immune system for defense, this includes production of Aβ42 peptide (11) and activation of the resident macrophages, microglia, resulting in neuroinflammation, neuronal loss and ultimately death (12). Unless constantly cleared, Aβ42 forms plaques, whilst toxic, soluble oligomeric forms also contribute to neuronal death. Familial forms of AD with mutations with increased Aβ42 formation, led to the "amyloid cascade hypothesis" (13) where amyloid precipitates the full spectrum of pathology and symptoms. Although clearly still very useful, this is undergoing re-appraisal in terms of the non-familial or common sporadic form (14, 15).

Whilst most cancers, including breast cancer, involve apparently unrestrained cell proliferation, AD involves cell loss. Neurones in the brain, are terminally differentiated post-mitotic cells, which if forced into cycle re-entry usually die (16). Cancer is associated with an increased glucose uptake by tumor cells, that is preferentially converted to lactate fermentation: a phenomenon known as the Warburg effect (17). The Warburg effect co-ordinates a number of cellular processes however, in addition to lactate fermentation, including preventing damage from reactive oxygen species (ROS), ensuring that cancer cells have a supportive microenvironment for cell proliferation (18). By contrast, AD is associated with an early reduction of glucose uptake and utilization in certain areas of the brain (19, 20). Due to its commonly seen insulin-resistance brain profile, AD is sometimes referred to as Type3 diabetes mellitus (T3DM) (19–22).

Despite the apparently different pathologies, we investigate here aspects of insulin/IGF signaling and the PI3K/Akt pathway that may determine these differences and briefly explore underlying commonalities between the mechanisms which play a role in the two disease states. Glucose intolerance increases generally with age (16, 17) and this is thought to be due to insulin-resistance, commonly observed in older adults (18, 19). Despite the opposing pathologies, cancer and AD have common risk factors such as aging, diabetes, obesity, smoking (23) and lack of exercise, each of which is also associated with insulin-resistance (24–27). Yet, as noted, although the AD brain often develops insulin-resistance, tumor cells generally do not. Here, we discuss normal cellular energy homeostasis and how this differs in cancer and AD.

REGULATION AND FUNCTION OF INSULIN AND IGF-1 IN HEALTH, CANCER AND AD

The main source of insulin is that secreted from the beta-cells of the pancreas in response to food; this normalizes the levels of blood glucose, by inducing its target tissues, liver, muscle, and fat cells to increase glucose uptake. IGF-I is secreted by the liver in response to growth hormone, and its circulating levels remain constant via its unique interaction with its IGF binding proteins (IGFBPs) (28). Unlike insulin, IGF-I (and IGF-II) are also made in most cells of the body, where they play key roles in growth, survival and metabolism. During an insulin-resistant state the usual normalizing processes are inhibited, leading to increased levels of circulating insulin and glucose. This also leads to a stimulation of hepatic IGF-I synthesis (29), and downregulation of IGFBPs-1 and−2, resulting in an increased bioavailability of IGF (30).

The phosphoinositide-3-kinase-(PI3K/Akt) signaling pathway, as depicted in **Figure 1A**, has been evolutionarily

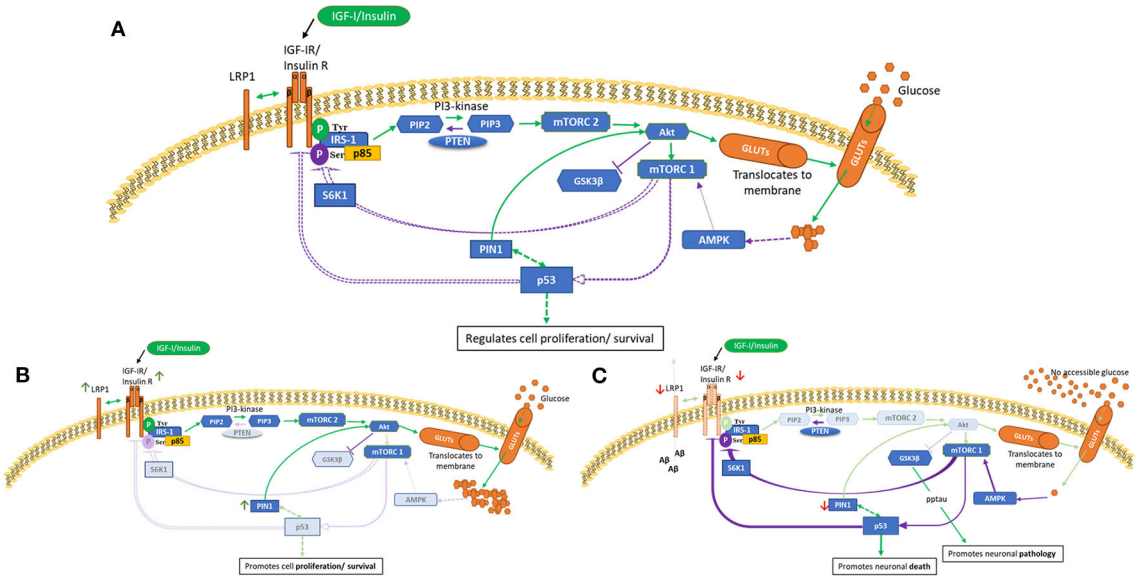

FIGURE 1 | PI3K/Akt pathway in health **(A)**, cancer **(B)** and AD brain **(C)** cells. This is a schematic of the PI3K/Akt cellular pathway which regulates cell proliferation, metabolism and death. These figures attempt to highlight possible differences in cancer and AD compared with health. These indicated differences, as described in human and animal tissues and in cell culture, are meant to represent general concepts not specific cases. **(A)** shows normal regulation **(B)** indicates a cancer phenotype **(C)** illustrates AD as an insulin-resistant state i.e., T3DM. Green lines represent activation and purple lines represent feedback from the activation pathway. Activation of the IGF-1/insulin receptors leads to tyrosine phosphorylation of IRS-1 and activation of mTORC2 and Akt, resulting in glucose uptake. Homeostasis is maintained partly by mTORC1 sensing of metabolic conditions, which, as appropriate, leads to phosphorylation of p53 and S6K1 serine phosphorylation of IRS-1. p53 is a negative regulator of IGF/insulin receptors, IGF-II and glucose transporters. [A] Normal cellular homeostasis as described above [B] In cancer, negative feedback pathways are switched off leading to upregulation of proliferation, metabolism and cell survival. A modified genetic landscape (e.g., p53, PTEN) enables tumor cells to benefit from a glucose-rich, IGF/insulin-rich environment (insulin-resistance such as in T2DM).In cancer, Akt can phosphorylate and inactivate GSK-3β, which results in increased protein synthesis that supports cell growth. [C] In AD brain with insulin-resistance, or if, due to decreased blood flow there is no glucose accessible, the PI3K/Akt pathway is effectively switched off or downregulated. This leads to upregulation of GSK-β that culminates in tau phosphorylation and aggregation and increased amyloid beta production. Lack of intraneuronal glucose would trigger AMPK to activate mTORC1, p53, S6K1 serine phosphorylation of IRS-1. This could be a self-perpetuating cycle.

conserved to regulate and maintain appropriate cell growth, survival and metabolism. This schematic presents an overview of glucose utilization management within normal cells. Two major activators of this pathway are insulin and IGFs (31) which act via specific receptor tyrosine kinases, IGF-IR and the insulin (IR) receptors. The IR can be spliced to produce two isoforms, IR-A and IR-B. Upon ligand binding, the receptors can dimerize forming IR/IGF-IR hybrids which have different biological consequences depending upon the IR isoform present (32, 33). Generally, insulin acts via the IR, and IGF-I and IGF-II act via the IGF-IR and hybrid receptors. IR-A binds IGF-II and insulin, whereas IR-B has a higher affinity for insulin (34, 35). Emerging data have expanded our understanding of the complexity of these receptors and how they signal, in terms of their localization, trafficking and their ability to interact with other molecules (36). To ensure adequate fuel, insulin/IGF-I bind and activate IR/IGF-IR, causing tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), leading to Akt activation. This results in translocation of glucose transporter isoforms (GLUTs) (37) to the cell membrane enabling glucose uptake. Phosphorylation of mTORC1 initiates subsequent negative feedback mechanisms, such as serine/threonine phosphorylation of IRS-1, which are lost in a cancer phenotype (**Figure 1B**). mTORC1 (as opposed to mTORC2) is also considered a main regulator of autophagy, that maintains tissue homeostasis by degrading "abnormal" cellular contents (38). Aberrant autophagy occurs in and contributes to both cancer and AD, however, the impact of this is dependent on the stage of disease for both pathologies (39, 40).

Epidemiologic studies have shown that "higher" normal levels of circulating IGF-I are associated with a 25% increased risk of breast cancer, compared with "lower" normal levels (41). Overexpression of the IGF ligands and their receptors, IGF-IR, IR (particularly IR-A) and IGF-IR/IR hybrid receptors leads to increased activity of the PI3K/Akt pathway (36, 42–44). The IGF-IIR is a single, non-signaling, transmembrane receptor, enabling homeostasis by clearing excess IGF-II (45); thus loss of function mutations in the IGF-II receptor (46, 47) and/or loss of IGF-II gene imprinting (48) can lead to excess IGF-II available to activate the PI3K/Akt pathway. IGFBPs are often deregulated in cancer; IGFBP-2, for example, is often upregulated which intrinsically downregulates phosphatase and tensin homolog (PTEN) (49, 50) removing the inhibitory brake on the PI3K/Akt pathway. The cells compensate by upregulating glucose transporters, notably GLUT1, which substantially increases glucose importation into the cytoplasm (51, 52) and the cells switch to lactate fermentation (Warburg effect).

AD as an insulin-resistant state, by contrast is exemplified in **Figure 1C**. The brain has a high energy dependence, using about 20% of the body's resting energy requirement (∼60% of glucose use) (53). Insulin crosses the BBB using a saturable transporter. Although GLUT1 and GLUT3 glucose transporters in the brain are insulin independent, the insulin dependent GLUT4 and GLUT8 are present in regions particularly affected in AD (54–56). IR (particularly IR-A) and IGF receptors are also strongly expressed in brain areas, such as the hippocampus, olfactory bulb, hypothalamus and cerebral cortex in neurones and glia and are important in memory formation in the hippocampus (55, 57, 58). Brain insulin and IGF levels are reduced in the aged brain with decreased insulin signaling and receptor activity (19, 59, 60), coinciding with decline in cognitive abilities. An early reduction of glucose uptake/metabolism is seen in pathology-related brain areas in AD and preclinical, pre-symptomatic subjects (61–63). Brain insulin-resistance is associated with impaired cognitive function (54) and is an important feature of AD in patients and in post-mortem tissue (64–69). Reduced insulin or IGF signaling leads to deficient uptake of glucose into neurones in those with mild cognitive impairment (MCI) who subsequently convert to AD, as well as being a major contributor to neuronal dysfunction and death in AD (70, 71). Reduced levels of insulin, IGF-I, II and their receptors associate with severity of pathology (19, 72). Furthermore, binding ability of these proteins is decreased, relative to increasing pathology (59, 73). In experimental studies, reduced IGF-I signaling was linked to increased deposition of Aβ (74, 75), phosphorylation of tau (76, 77), increased oxidative stress, neuro-inflammation and neuronal death (78). Of interest also, is the finding that the (non-toxic) monomeric form of Aβ can activate insulin/IGF-1 receptor signaling, and since these monomers aggregate in early AD, it is suggested that this may form a prelude to the disease process (79). Notably, systemic administration of IGF-I was able to lower the toxicity of Aβ in normal mice (80) and restore cognitive function in AD mouse models (81).

There are studies which are not in line with the hypothesis that IGF-I downregulation in AD is causative in the disease process but rather may be protective. The mixed results may partly lie in the fact that total IGF-I poorly reflects its bioactivity as most circulating IGF-I is bound to IGFBPs and will therefore be biologically inactive (82). There are also several variables between studies, for instance age of onset, stage of disease progression, presence of diabetes, or IGF-I gene polymorphisms.

Therefore, overall, in cancer and AD, the control of these pathways is compromised, allowing feed-forward and feed-backward cycles which lead either to cell over proliferation/deregulation or conversely death.

COMPARING REGULATORY MOLECULES AND THEIR ROLE IN AD AND CANCER

The PI3K/Akt pathway is kept in equilibrium by key regulators, some of these are briefly discussed here in terms of their effects on glucose metabolism in cancer and AD and are depicted in **Figures 1A–C**.

IRS-1

IRS-1 plays a critical regulatory role in transmitting signals from IGF-IR/IR receptors via the PI3K/AKT pathway. It is commonly overexpressed in cancer and this has been associated with poor outcome for breast cancer patients (83), particularly if the tumor is positive for the estrogen receptor (84). Tyrosine phosphorylation activates and serine/threonine phosphorylation inhibits IRS-1 activity. Ribosomal protein S6 kinase beta-1 (S6K1) is one kinase responsible for inhibitory phosphorylation of IRS-1(85) and this negative feedback inhibition is lost in many cancers, including breast cancer (86).

In AD, insulin and IGF signaling is adversely affected in important brain areas. Phosphorylation of IRS-1 at serine 616 (pS616) and p-serine 636/639 are early markers of brain insulin-resistance, commonly present in MCI and AD (67). Aβ oligomers are thought to initiate IGF-I resistance and IRS-1 inactivation and to be associated with increased oligomeric Aβ plaques and memory impairment. Neurones in the temporal cortex in AD have been reported to show reduced levels of active IRS-1 and−2, but increased inactivated IRS-1, particularly at p-serine 312 and 616, and this was associated with NFT (73). Apart from indicating insulin-resistance and decreased glucose uptake, it suggests a relationship between IRS-1, tau (NFT) and Aβ pathology.

p53 Tumor Suppressor Gene

Wild-type p53 regulates many cell functions including cell cycle arrest, apoptosis and metabolism (87). P53 negatively regulates IGF-IR, IGF-II, GLUTs 1 and 4 and positively stimulates IGFBP-3 (pro-apoptotic factor) (88–91). In cancer, including breast cancer, p53 is often mutated, resulting in a loss of its tumor suppressor activity (92–94). This disrupts regulation of IGF-IR, IGF-II, GLUTs 1, 4, and IGFBP-3, leading to enhanced activation of the PI3K/Akt pathway and glucose uptake. Increased Aβ positively correlates with p53 levels (91, 92). AD brain levels of p53 are thus increased, which promotes tau hyperphosphorylation and ultimately neuronal death (90).

Peptidyl-Prolyl Cis-Trans Isomerase NIMA-Interacting-1 (PIN1)

Pin1 is a peptidyl-prolyl cis–trans isomerase (PPIase) able to isomerise p-serine/p-threonine-proline sequences thus effecting conformational change which alters the activity of its target proteins (95). It is highly expressed in many cancers (96, 97) and facilitates activation of the PI3K/Akt pathway. One way it does this is by increasing Akt stability through serine 473 phosphorylation (98). In breast cancer, high levels of both Akt-p-S473 and PIN1 predict a poorer prognosis than either alone (99). PIN1 can also induce a conformational change to the tumor suppressor gene p53 (100) and its overexpression in the presence of p53 mutations are prognostic for poor clinical outcome in breast cancer (101). SUMO protease-1 (SENP1) binds to, and deSUMOylates PIN1, and its levels correlate with those of PIN1 in breast cancer (102, 103). PIN1 is inhibited by *BRCA-1*, the tumor suppressor

gene (104) suggesting that PIN1 would play an important role in the development of tumors in which *BRCA1* is mutated. PIN1 also supports increased cell proliferation by promoting glycolysis in tumor cells. This is achieved by stimulation of pyruvate kinase translocation (that catalyses the rate-limiting step during glycolysis) to the nucleus (95, 105). As a consequence of these functions, PIN1 inhibitors have been developed and shown to slow the progression of cancer (96).

In brain, PIN1 is located in neuronal dendrites and postsynaptic densities and its activity and expression are reduced in MCI and AD (106, 107), likely to make neurons more vulnerable to Aβ and increasing synaptic degeneration (108). Notably, PIN1 enables tau dephosphorylation via protein phosphatase PP2A and co-localizes with hyperphosphorylated tau in AD brain (109).

Low-Density Lipoprotein Receptor–Related Protein 1 (LRP1)

The LRP1 receptor is a multifunctional receptor involved in many cellular functions including endocytosis and cell signaling. Notable is its intrinsic link with energy homeostasis; through its binding to the IGF-IR (110) and the IR (111), LRP1 plays a central role in insulin/IGF signaling affecting cell proliferation, survival, glucose and lipoprotein metabolism (112, 113).

The role that LRP1 plays in cancer is dependent upon the type of tumor and the cellular environment. In breast cancer, early reports indicated that a low expression of LRP1 correlated with more aggressive tumors (114). More recent work, however, consistently indicates a role for LRP1 in supporting breast cancer cell invasion and metastasis (115, 116) by increasing expression of matrix metalloproteinases (MMPs), MMP-2, and 9 (117).

In the brain, LRP1 is important for cell survival, lipoprotein metabolism and synaptic plasticity, and is highly expressed in neurones. It binds leptin, enabling leptin receptor phosphorylation and Stat3 activation. Deletion of the *Lrp1* gene in the mouse hypothalamus results in increased body weight (obesity) (118); conditional *Lrp1* brain knock-out produces glucose intolerance (111). LRP1 interacts with the insulin receptor, regulating insulin signaling and glucose uptake, and influencing GLUT3 and−4 glucose transporter levels (111). Insulin resistance in peripheral tissues in rodents involves loss of GLUT4 function (119, 120). Centrally, in the rat hippocampus, GLUT4 is vital to memory acquisition, inhibition causing memory impairment (56). Amyloid requires constant clearance pathways, LRP1 is known for its function as a clearance receptor able to remove amyloid across the BBB (121), but also to endocytose Aβ for elimination by lysosomes. LRP1 expression is reduced with age in mouse (122) and human brain (123), and to a greater degree in AD (122, 123). Notably, hyperglycaemia and increased insulin resistance, as in type-2 diabetes mellitus (T2DM), suppress LRP1 expression and exacerbate AD pathology in mice (111). Reduced LRP1 levels are associated with increased neuronal death (124) signifying that LRP1 is required for the neuroprotective effects of insulin signaling (125).

SUMMARY

The PI3K/Akt pathway is central to the sensing of metabolic and nutritional changes in our environment and is clearly deregulated in both cancer and AD. Considering that most of the risk factors for both, such as obesity, T2DM and smoking are modifiable through lifestyle changes, an effective strategy could be a preventive approach; for instance re-establishing physiological glucose levels by diet. This minireview, however, attempts to briefly explore some of the underlying mechanisms to identify possible therapeutic targets for these conditions, already ongoing. By addressing the apparent inverse relationship between cancer and AD we hope to identify regulatory molecules in the PI3K/Akt pathway important in cell proliferation and glucose utilization. In cancer this leads to upregulation of glucose uptake and cell proliferation, which contrasts with AD where there is lack of glucose availability, increased pathology, and consequent neuronal death. For both breast cancer and AD there has been a drive for the identification of biomarkers for early detection, ultimately to improve long-term survival. Notably, pre-clinical studies have identified IRS-1, p53, PIN1 and LRP1 as individual potential therapeutic targets (126–133) for both disease states, and changes in these are in themselves putative biomarkers.

These may provide alternative targets for future trials, but the possibility of inverse effects of altering these proteins, as we outline here, suggests that a delicate balance is required within the PI3K/Akt pathway. It is notable therefore that Metformin, an antihyperglycemic agent for diabetes, appears to promise some beneficial therapeutic outcome in both cancer and AD (134, 135). In cancer the mechanism is likely to be via mTOR inhibition and activation of p53 (136); in T2DM and T3DM-AD, it is probably the reduction of insulin-resistance (137). Whilst it is challenging to develop specific drugs for the clinical setting, understanding the regulatory aspects of this pathway may enable a co-targeting approach to reduce non-specific toxicity and increase specificity, thus achieving a better outcome.

AUTHOR CONTRIBUTIONS

SA-B and CP proposed the concept for the review. CP, SA-B, KB, JH and RB contributed to writing the paper. KB designed the figures. All authors contributed to the article and approved the submitted version.

FUNDING

We acknowledge support from Cancer Research UK (C18281/A19169) Programme Grant (The Integrative Cancer Epidemiology Programme); BRACE (Bristol Research into Alzheimer's and Care of the Elderly). SA-B is a Sigmund Gestetner Senior Research Fellow. The research was also supported by the NIHR Bristol Nutrition Biomedical Research Unit based at University Hospitals Bristol NHS Foundation Trust and the University of Bristol.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 02 September 2020
doi: 10.3389/fendo.2020.00614

Pregnancy-Associated Plasma Protein-A2 Is Associated With Mortality in Patients With Lung Cancer

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OPEN ACCESS

Edited by:
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Reviewed by:
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Julian Christians, Simon Fraser University, Canada

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *22 June 2020*
Accepted: *28 July 2020*
Published: *02 September 2020*

Citation:
Hjortebjerg R, Espelund U, Rasmussen TR, Folkersen B, Steiniche T, Georgsen JB, Oxvig C and Frystyk J (2020) Pregnancy-Associated Plasma Protein-A2 Is Associated With Mortality in Patients With Lung Cancer. Front. Endocrinol. 11:614. doi: 10.3389/fendo.2020.00614

Pregnancy-associated plasma protein-A (PAPP-A) and its homolog PAPP-A2 are enzymes that modulate the availability and mitogenic activity of insulin-like growth factor-I (IGF-I). PAPP-A has been implicated in numerous cancers but reports on PAPP-A2 in malignancy are non-existent. In a prospective observational study of 689 patients under suspicion of lung cancer, we examined levels of PAPP-A and PAPP-A2 and their relationship with mortality. Serum PAPP-A and PAPP-A2 concentrations were determined in pre-diagnostic blood samples using ELISA, and immunohistochemical staining of PAPP-A and PAPP-A2 was performed in malignant tissue from five operable patients. A total of 144 patients were diagnosed with lung cancer, whereas the diagnosis was rejected in 545 subjects, who served as a control group. PAPP-A2 concentrations were higher in patients with lung cancer [median (IQR): 0.33 (0.21–0.56) ng/mL] than in controls [0.27 (0.17–0.39) ng/mL], $p < 0.001$, whereas PAPP-A levels did not differ. Presence of PAPP-A and PAPP-A2 were confirmed in tumor specimens, and staining occurred in a heterogeneous pattern. Patients were observed for a median (range) of 7 (6; 8) years, during which 114 patients (79.2%) died. Patient mortality differed according to PAPP-A2 tertile ($p < 0.001$). PAPP-A2 was associated with mortality with an unadjusted hazard ratio (95% CI) per doubling in protein concentration of 1.30 (1.12; 1.53), $p = 0.001$. In a multivariable model adjusted for age, sex, and BMI, PAPP-A2 remained predictive of the endpoint with a hazard ratio per doubling in protein concentration of 1.25 (1.05; 1.48), $p = 0.013$. Collectively, PAPP-A2, but not PAPP-A, is elevated in patients with lung cancer and associated with mortality. This novel role of PAPP-A2 in cancer warrants further functional studies as well as validation in external cohorts.

Keywords: insulin-like growth factor, insulin-like growth factor binding protein, lung cancer, mortality, pregnancy-associated plasma protein-A, pregnancy-associated plasma protein-A2

INTRODUCTION

Lung cancer is one of the most common human malignancies worldwide with considerable attendant societal costs. Tumor heterogeneity and the lack of seromarkers for detection of the disease at early stages pose a formidable challenge and contribute to high mortality rates. Insulin-like growth factor I (IGF-I) is a pivotal player in the multifaceted process of malignant disease, including lung cancer, and signaling through the IGF-I receptor (IGF-IR) stimulates mitogenesis, metabolism, and anti-apoptosis (1, 2).

Pregnancy-associated plasma protein-A (PAPP-A) and PAPP-A2 comprise the only two known members of the pappalysin family of metalloproteinases, sharing 45% amino acid identity (3, 4). They are responsible for proteolytic cleavage of a subset of IGF-binding proteins (IGFBPs), through which they increase IGF availability and potentiate its growth stimulatory effects (5). PAPP-A has been suggested as an accomplice in several types of cancer (6–9) and has been extensively studied due to its biomarker potential (3, 10–13). Although PAPP-A2 was recently established as a regulator of the IGF axis in human physiology (14), the biology of PAPP-A2 is poorly understood compared to PAPP-A (15), and there are currently no reports linking PAPP-A2 protein and cancer mortality (9).

PAPP-A specifically cleaves IGFBP-2,−4, and−5 and is widely expressed in multiple tissues, including those of tumor origin, where it tethers to cell surfaces (16, 17). Thus, PAPP-A causes a release of bioactive IGF in close proximity to the IGF-IR. Shifts in PAPP-A levels have been suggested to modify the relationship between bound and free IGF in various neoplasms (8, 18–20). In patients with lung cancer, serum PAPP-A levels have been shown to be elevated (19), and down-regulation of PAPP-A expression decreases lung cancer progression *in vivo* (21). The present authors previously described a cohort of women with ovarian cancer, in which PAPP-A levels were investigated in serum and malignant ascites (20). In ascites, which surrounds the ovarian tumor in the abdominal cavity and is a negative prognostic factor, PAPP-A levels were 46-fold higher as compared to serum from the same patient. It was further shown that the ability of ascites to activate the IGF-IR *in vitro* was increased by 31% as compared to serum, and immunohistochemistry (IHC) of ovarian tumor specimens revealed abundant staining of both IGF-IR and PAPP-A.

Similar to PAPP-A, placentally derived PAPP-A2 is abundantly present in the circulation throughout pregnancy, but the protein is also detectable in non-pregnant men and women (22). However, PAPP-A2 has generally not been investigated in human pathologic conditions outside pregnancy. PAPP-A2 exhibits proteolytic activity against IGFBP-3 and−5, but unlike PAPP-A, PAPP-A2 does not show surface tethering (15). Recently, Dauber et al. (14) reported the first human PAPP-A2 deficiency cases, who presented with short stature and severe perturbations in the IGF system. This finding provided conclusive evidence of the importance of PAPP-A2 in human physiology.

The present study evaluated PAPP-A and PAPP-A2 levels in serum from 689 patients under suspicion of lung cancer and assessed PAPP-A and PAPP-A2 expression by IHC in surgical specimens. Furthermore, we investigated the associations of PAPP-A and PAPP-A2 with mortality in the 144 patients with a cancer diagnosis and compared their prognostic performances.

METHODS

Patient Characteristics

The Department of Pulmonary Medicine at Aarhus University Hospital receives patients under suspicion of lung cancer referred from their general practitioner or other hospital departments within the region of Aarhus, Denmark. All referred patients are examined in a fast-track diagnostic setup, where medical examination, routine biochemistry, CT, PET, lung function tests, endosonography, and biopsies are performed within four weeks of their first visit.

All patients referred from February 2009 through April 2011 were invited to participate in the present study at their first visit. A total of 1,405 patients were registered, and information was obtained on smoking habits, symptoms including dyspnea, height, weight, recent weight gain or loss, and reasons for exclusion when applicable. These data were paired with routine biochemistry, diagnoses given at the end of the diagnostic course, lung function tests, diagnoses given in conjunction with previous contacts with the Danish health system, and Charlson comorbidity index. The TNM system was used to stage the cancer; T describing the size of the primary lung tumor, N describing regional lymph node involvement and M describing distant metastasis. These values were combined to assign an overall cancer stage (1–4). Project blood samples were collected in conjunction with the routine samples, centrifuged, separated in aliquots, and stored at −80° until assay. Exclusion criteria were previous malignancies apart from non-melanoma skin cancer (n = 188), severe heart failure (NYHA III/IV) (n = 11), thyroid dysregulation (n = 67), lack of mental resources (23), linguistic and cultural barriers (n = 45) and long dwell time (n = 19). Of all registered cases in the period, 132 patients were not asked by the investigator. In addition, biochemical criteria were applied to identify patients with poorly managed diabetes and decreased renal function. Patients with diabetes and an HbA1c above 7.0% DCCT were excluded from the study (calculated as an average of all available measurements across the study period) (n = 123). This level equals 53 mmol/mol by the IFCC standard and reflects an estimated average plasma glucose of 8.5 mmol/l. Patients with an estimated glomerular filtration rate (eGFR) below 40 ml/min were excluded (determined by the MDRD formula without correction for ethnicity) (n = 28). Exclusion criteria reduced the cohort to 803 patients. Of these, 35 did not wish to participate. Some patients were subsequently diagnosed with malignant mesothelioma (n = 12) and other cancers than lung cancer including metastases (n = 36), and these two groups were excluded from analyses. Finally, patients were also excluded due to lack of sufficient blood for determination of target proteins (n = 15), resulting in a total of 705 patients available for laboratory measurements. However, to ensure that all variables were investigated in the context of lung cancer,

patients were excluded if a new cancer diagnosis occurred <2 years from study inclusion ($n = 16$). Patients that received a new cancer diagnosis more than 2 years into follow-up were included in the study ($n = 34$). A total of 689 patients were eligible for analysis (**Figure 1**). Hereof, 144 patients (20.9%) were diagnosed with lung cancer, whereas this diagnosis was rejected in 545 patients. Patients were grouped as follows: controls (control, $n = 545$), small cell lung carcinoma (SCLC, $n = 13$), non-small cell lung carcinoma, NSCLC, adenocarcinoma subtype (NS-Ad, $n = 75$), NSCLC, squamous cell subtype (NS-Sq, $n = 27$), and NSCLC, other subtypes than NS-Ad and NS-Sq (NS-x, $n = 29$).

Baseline descriptions of controls and patients are summarized in **Table 1**, including the clinical stage in lung cancer patients. Patients were allocated to treatment independent of study participation. A total of 117 controls were diagnosed with pneumonia, hemoptysis, sarcoidosis, or other respiratory diseases. Control subjects where a malignant cause was ruled out were given an unspecific "observation" diagnosis ($n = 303$), whereas others showed an abnormal computerized tomography scan ($n = 182$). Written informed consent was obtained from all patients, and the study (id: 1-10-72-155-12) was approved by the Central Denmark Region Committees on Biomedical Research Ethics (IRB 0005129). The study was conducted in accordance with the Declaration of Helsinki.

Outcome Measures

All-cause mortality for lung cancer patients was recorded until March 2017. The 144 patients with a cancer diagnosis had a median (range) survival of 377 days (14 days−8 years). Since the first cancer patient was included in March 2009 and the final patient in March 2011, all cancer patients were followed for at least 6 years [median (range): 7 (6; 8) years]. Survival was documented for each individual using the Danish Civil Registration System and the National Causes of Death Registry, which offers information from physicians on causes of death according to the International Classification of Diseases, Tenth Revision (ICD-10). Because of the high-quality Danish registration system, no patients were lost during follow-up.

Laboratory Measurements

Routine biochemistry was performed at the hospital's laboratory using widely available automated assays. Serum protein levels

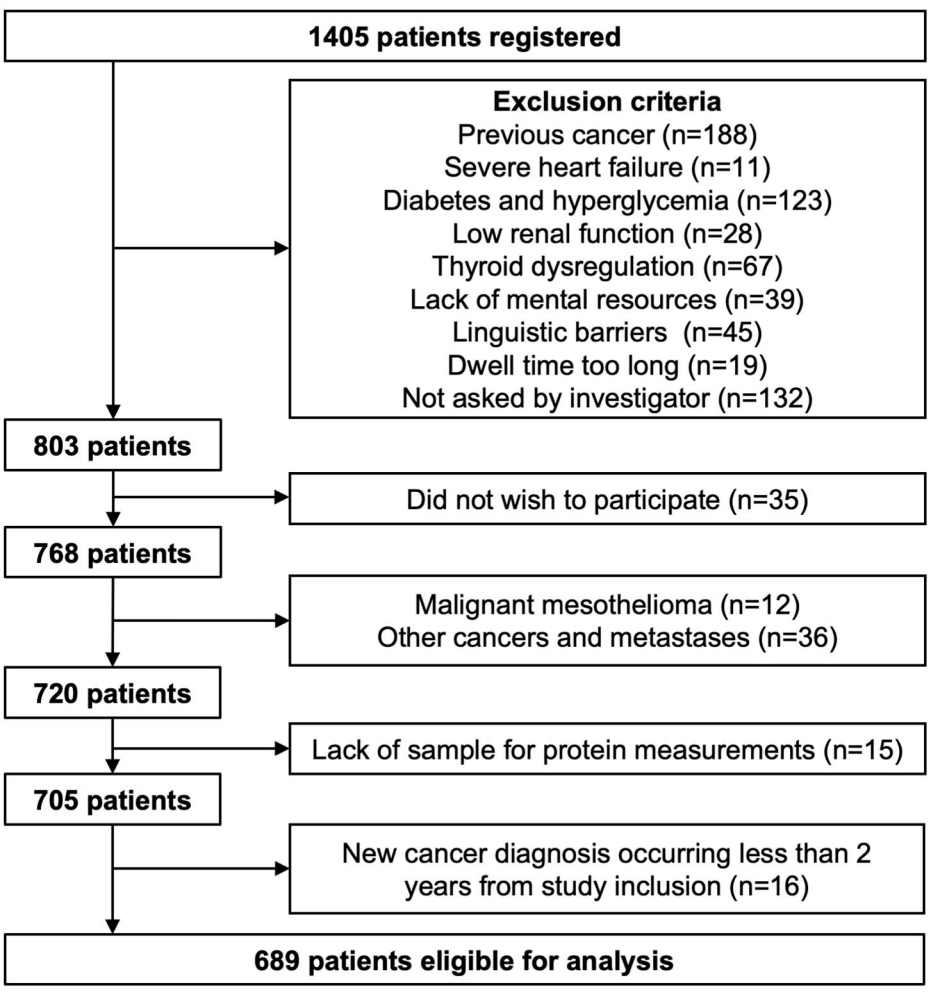

FIGURE 1 | Flow chart of patient inclusion. Some patients met more than one exclusion criteria; hence the sum does not equal the reduction in patient number.

TABLE 1 | Baseline and survival characteristics.

Characteristics	Control	Cancer	Cancer subtype			
			SCLC	NS-Ad	NS-Sq	NS-x
Number, *n*	545	144	13	75	27	29
Males, *n* (%)	266 (48.8)	66 (45.8)	6 (42.2)	23 (30.7)**	18 (66.7)	19 (65.5)
Age, years	61.9 ± 13.1	67.1 ± 10.6**	67.7 ± 10.9	64.7 ± 10.0	72.8 ± 9.6**	67.5 ± 11.4*
BMI, kg/m^2	25.5 ± 4.9	23.6 ± 3.7**	25.6 ± 3.6	23.1 ± 3.5**	23.9 ± 3.6	23.5 ± 4.4*
CRP (mg/L)	2.6 (0.9; 6.8)	13.9 (3.6; 32.9)**	5.8 (2.4; 22.8)*	8.7 (3.1; 24.0)**	32.5 (20.6; 48.7)**	13.1 (4.4; 33.9)**
eGFR	85 (74; 99)	90 (79; 106)**	86 (81; 101)	88 (78; 103)	91 (79; 112)	93 (83; 108)
Smoking status, *n* (%)						
Never	117 (21.5)	7 (4.6)	0 (0.0)	4 (5.3)	1 (3.7)	2 (6.9)
Former/current	368 (67.5)	131 (91.0)	13 (100.0)	69 (92.0)	25 (92.6)	24 (82.8)
Unknown/missing	60 (11.0)	6 (4.2)	0 (0.0)	2 (2.7)	1 (3.7)	3 (10.3)
Stage, *n* (%)						
1		27 (18.8)	2 (15.4)	17 (22.7)	2 (4.4)	6 (20.7)
2		14 (9.7)	0 (0.0)	7 (9.3)	4 (14.8)	3 (10.3)
3		29 (20.1)	5 (38.5)	6 (8.0)	13 (48.2)	5 (17.2)
4		56 (38.9)	4 (30.8)	35 (46.7)	5 (18.5)	12 (41.4)
Unknown		18 (12.5)	2 (15.4)	10 (13.3)	3 (11.1)	3 (10.3)
PAPP-A, ng/mL	1.03 (0.84; 1.27)	1.04 (0.86; 1.36)	1.02 (0.90; 1.54)	1.01 (0.84; 1.26)	1.10 (0.78; 1.43)	1.02 (0.90; 1.26)
PAPP-A2, ng/mL	0.27 (0.17; 0.39)	0.33 (0.21; 0.56)**	0.33 (0.22; 0.78)	0.29 (0.20; 0.47)	0.47 (0.24; 0.67)**	0.34 (0.23; 0.49)*
Survival, days		377 (190; 1,301)	550 (324; 1,717)	397 (206; 1,535)	281 (141; 899)	307 (157; 865)
Mortality at endpoint, *n* (%)	78 (14.3)	114 (79.2)	10 (76.9)	59 (78.7)	23 (85.2)	22 (75.9)

*Baseline and survival characteristics in controls, patients with lung cancer and subtypes of lung cancer. Patients are grouped as follows; control subjects (Control), all cancer patients, (Cancer), small cell lung carcinoma (SCLC), non-small cell lung carcinoma (NSCLC), NSCLC adenocarcinoma subtype (NS-Ad), NSCLC squamous cell subtype (NS-Sq), and NSCLC other subtypes than NS-Ad and NS-Sq (NS-x). Survival refers to median survival time and comprises all cancer patients who were censored or experienced an event. Categorical variables are indicated as number (n) and percentage (%) of patients, and continuous variables are mean ± SD or median (25th percentile; 75th percentile). *$p < 0.05$, **$p < 0.005$ as compared to controls. BMI, body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; PAPP-A, pregnancy-associated plasma protein-A.*

of PAPP-A and PAPP-A2 were measured using PAPP-A (AL-101) and PAPP-A2 (AL-109) ELISA kits (AnshLabs, Webster, TX, USA). All samples were analyzed in a blinded fashion in random order.

Immunohistochemistry

A subgroup of five patients who were operable provided tumor tissue for IHC, which was performed as previously described (20) using antibodies specific for PAPP-A (PAC1-D8-mIgG2a) (24) and PAPP-A2 (P257) (22) at 10 and 20 mg/L, respectively.

Statistical Analysis

The assumption of normality was checked using quantile-quantile plots and by the Shapiro-Wilk test, and non-normally distributed variables were transformed prior to statistical analyses. Whenever possible, parametric statistical tests were applied. Groups were compared with Student's *t*-test (two groups) or one-way ANOVA and *post-hoc* tests with Bonferroni's correction (multiple groups). If there was evidence against the assumption of equal variance by Bartlett's test, or if data did not follow a normal distribution, Wilcoxon rank-sum test or Kruskal-Wallis test was applied, respectively. Categorical variables were evaluated by χ^2-test. PAPP-A and PAPP-A2 were modeled categorically as tertiles and as continuous variables after log transformation using log(protein)/log(2). Accordingly, one unit increase in protein level on the $\log_2$-scale corresponds to a doubling in protein. Test for linear trend (continuous protein level) across ordered groups (cancer stage) was performed by linear regression analyses with the ordered group as a continuous explanatory variable with equal distance between steps. Test for ordered categorical trend (protein tertile) across ordered groups was performed using an extension of the Wilcoxon rank-sum test developed by Cuzick (25).

The area under the receiver operating characteristic (ROC) curve (AUC) was used to assess the prognostic ability of PAPP-A and PAPP-A2. However, AUC is a metric for binary classification and does not consider individual survival times and censoring. As proposed by Harrell et al. (26) as an extension of AUC, the concordance (C) index was used as a measure of concordance between the protein of interest and the possibly censored survival outcome, using a similar range from 0 to 1. Suggested by Pencina et al. (27, 28), the Harrell's C index is the most appropriate in capturing the discriminating ability of a prognostic variable to separate subjects with varying survival time and outcome status.

Kaplan-Meier survival curves were performed for PAPP-A and PAPP-A2 tertiles, and incidence distributions were compared using the log-rank test. Cox proportional hazards models were developed to explore associations between survival endpoint and the explanatory variable, using both the continuous variable and tertiles with the low tertile as reference group. Hazard ratios

(HRs) assessed the risk of death in unadjusted models and after adjustment for *a priori* defined covariables; age, sex, and BMI. Smoking status was not associated with PAPP-A or PAPP-A2 level, and hence, not included. The validity of the proportional hazards and linearity assumptions were checked by log-log plots, fitted survival curves and smoothed martingale and Schonenfeld residuals plot; no deviations from proportionality were identified (26, 29, 30).

Results are presented as mean ± SD for normally distributed data and median (25th percentile; 75th percentile) for skewed data. AUC, C-statistics, and HRs are presented with 95% confidence intervals (CI). Two-tailed *P*-values < 0.05 were considered statistically significant. Data were analyzed using Stata software (StataCorp LP, College Station, TX, USA). Harrell's C index and Somers' D statistics for censored data was calculated using the "somersd" module in Stata version 13.

RESULTS

Baseline Characteristics

Control and patient characteristics by subtype of lung cancer are given in **Table 1**. Cancer patients were significantly older than controls and had a lower BMI, and a higher percentage were current or former smokers. Among cancer subtypes, patients were similar with regards to age and BMI, and disease stage did not differ between cancer subgroups. However, as expected, cancer patients differed from controls in a wide range of inflammatory markers, organ markers, and markers of general nutritional status (data not shown). Especially patients with squamous NSCLC exhibited a distinct pattern in their biochemical profile, suggesting a higher degree of inflammation than in patients with other subtypes.

To assess if the included cases were representative of the total group of patients diagnosed with lung cancer in the study period, patients were compared to non-participating patients. The distribution of non-included and included patients on the various histological diagnoses as well as disease stages did not differ (data not shown).

PAPP-A2, but Not PAPP-A, Is Elevated in Patients With Lung Cancer and Differ Between Subtypes

Serum PAPP-A2 was elevated in patients with lung cancer as compared to control subjects ($p < 0.001$) (**Table 1**). When comparing the individual cancer subtypes with controls, the highest concentrations were observed in the NS-Sq group ($p < 0.001$). Levels in the NS-x group were also elevated ($p < 0.05$), whereas PAPP-A2 was not significantly higher in the other cancer subtypes. By contrast, PAPP-A levels were similar in cancer and control subjects and did not differ among cancer subtype or stage. PAPP-A2 as a continuous variable was not associated with cancer stage ($p = 0.123$), and thus, patients with early-stage cancer displayed similar concentrations as patients with more advanced-stage cancer. However, when assessing PAPP-A2 tertiles, a higher tertile was significantly associated with higher cancer stage ($p_{trend} = 0.003$). Cancer stage according to PAPP-A and PAPP-A2 tertiles is shown in **Figure 2**.

The group of NS-Ad patients was of sufficient size to allow for further analyses of cancer TNM classification. Overall group differences as well as linear trend across TNM category were assessed. However, neither PAPP-A nor PAPP-A2 was associated with tumor size, lymph node involvement or metastatic status.

PAPP-A was positively associated with age ($r = 0.27$, $p < 0.001$) in all subjects, whereas PAPP-A2 was positively associated with age ($r = 0.40$, $p < 0.001$) and negatively associated with BMI ($r = -0.19$, $p < 0.001$). Additionally, in the cancer patients, PAPP-A2 showed correlations with several markers of inflammation, organ status and overall illness, including C-reactive protein (CRP) ($r = 0.34$, $p < 0.001$), erythrocyte sedimentation rate (ESR) ($r = 0.29$, $p < 0.001$), eGFR ($r = -0.18$, $p < 0.05$) and hemoglobin ($r = -0.30$, $p < 0.001$). PAPP-A2 was also positively associated with levels of leukocytes, neutrophils, and monocytes (all $p < 0.05$).

Immunohistochemistry

The expression of PAPP-A and PAPP-A2 was confirmed by IHC of tumors removed during surgery. Tissue originated from one patient with SCLC, two with NS-Ad, and two with NS-Sq. The anti-PAPP-A antibody stained tumor specimens in a vacuole-like or cell membrane accentuated pattern, as expected for a secreted protease, and staining intensity varied across cell types and between patients. Staining for PAPP-A2 was demonstrated in four out of five patients, with no staining in the SCLC tumor sample. PAPP-A2 staining was present in malignant cells as well as areas densely infiltrated by macrophages. Staining was mild to moderate and occurred in a heterogeneous pattern. Examples of the breadth and intensities of PAPP-A2 staining patterns are illustrated in **Figure 3**.

Survival Analyses of Cancer Patients

During follow-up, 114 patients (79.2%) died, and median (range) survival of all cancer patients was 377 days (14 days−8 years) (**Table 1**). Median survival of patients was 550 days in SCLC, 397 days in NS-Ad, 281 days in NS-Sq and 307 days in NS-x. There was no difference in overall mortality between cancer subtypes; 76.9% in SCLC, 78.7% in NS-Ad, 85.2% in NS-Sq and 75.9% in NS-x ($p = 0.672$).

ROC AUC was 0.55 (0.43; 0.67) for PAPP-A and 0.63 (0.52; 0.73) for PAPP-A2. Log-rank analysis showed similar mortality in the low, middle and high PAPP-A tertiles ($p = 0.324$). However, incidence distributions differed significantly according to PAPP-A2 tertile ($p < 0.001$), and mortality increased with increasing PAPP-A2 tertile ($p_{trend} < 0.001$). Mortality distribution in the PAPP-A2 tertiles did not differ among the various lung cancer subtypes ($p = 0.341$). Log-rank test and deaths among the various lung cancer subtypes are shown in **Table 2**. To suggest and illustrate a future potential clinical utility of PAPP-A and PAPP-A2 as biomarkers, Kaplan-Meier survival curves were constructed according to tertiles of PAPP-A and PAPP-A2 (**Figure 4**). To further investigate the prognostic power of PAPP-A and PAPP-A2, we calculated Harrell's C index, which assesses discrimination ability of survival models. Harrell's C index for

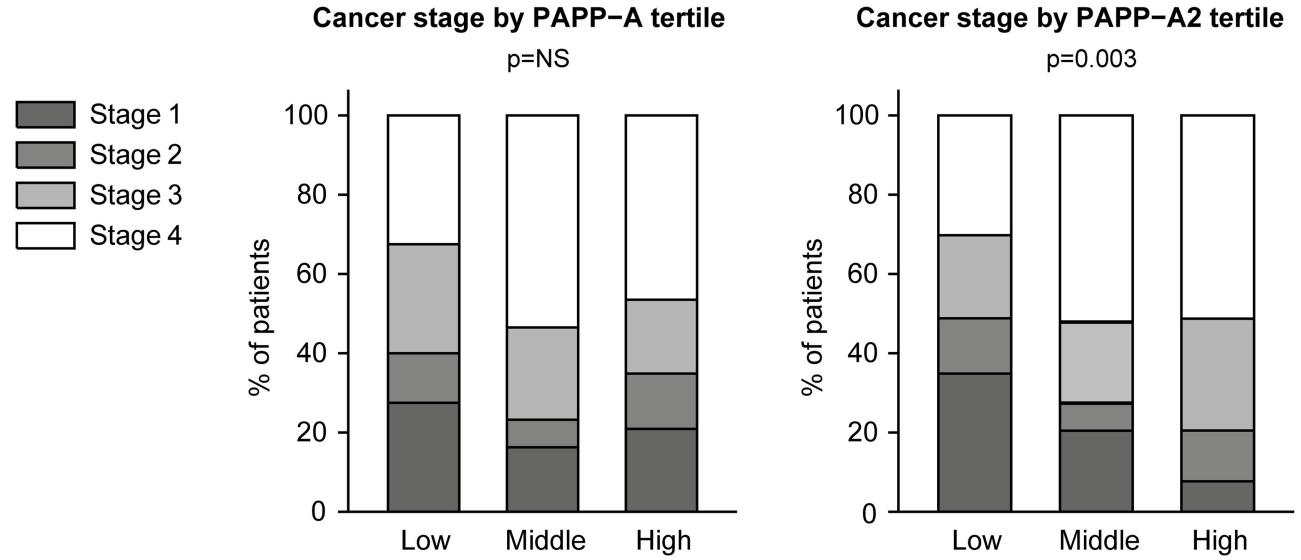

FIGURE 2 | Cancer stage according to PAPP-A and PAPP-A2 tertiles. PAPP-A, pregnancy-associated plasma protein-A.

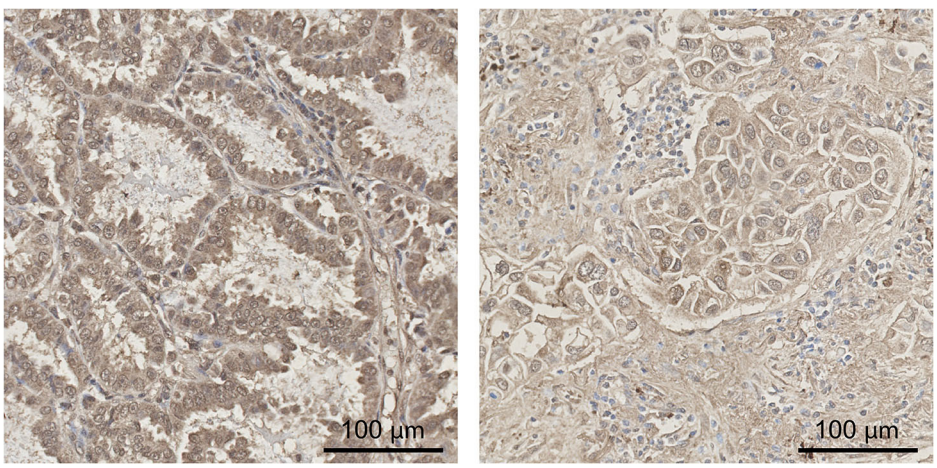

FIGURE 3 | Immunohistochemical staining of PAPP-A2. Expression of PAPP-A2 in lung cancer tissue was determined by immunohistochemical staining. Examples are shown for two patients with non-small cell lung cancer of adenocarcinoma subtype. PAPP-A2 staining was present in malignant cells in recognizable glandular patterns as well as areas densely infiltrated by macrophages. Staining was moderate (left) and weak (right) and occurred in a heterogeneous pattern. Scale bar = 100 µm. PAPP-A2, pregnancy-associated plasma protein-A2.

PAPP-A was 0.52 (0.46; 58), whereas that for PAPP-A2 was 0.62 (0.57; 0.68).

The association between mortality and PAPP-A or PAPP-A2 was investigated using both the continuous variable and tertiles with the low tertile as reference group (**Table 3**). PAPP-A was not associated with outcome. In contrast, with each 2-fold increase in PAPP-A2, the mortality increased by 30% [HR: 1.30 (1.12; 1.53), $p = 0.001$]. In a categorical model using the first tertile as reference, PAPP-A2 was associated with mortality with a HR of 1.57 (0.98; 2.50), $p = 0.060$, for the second tertile and 2.60 (1.64; 4.14), $p < 0.001$, for the third tertile. In multivariable Cox regressions adjusted for age, sex, and BMI, PAPP-A2 as a continuous variable remained predictive of the endpoint, whereas PAPP-A2 as a categorical variable remained significant when the high tertile was compared to the low tertile.

DISCUSSION

This prospective study sought to investigate PAPP-A and PAPP-A2 in patients with lung cancer and evaluate potential associations with mortality. PAPP-A2, but not PAPP-A, was elevated in patients with lung cancer, and we demonstrated a prognostic significance of PAPP-A2. The present study is the first exploration of the potential clinical significance of PAPP-A2 in

TABLE 2 | Log-rank analyses on all-cause mortality according to PAPP-A or PAPP-A2 tertiles.

Log-rank	Concentration (ng/mL)	Total patients (n)	SCLC (n)	NS-Ad (n)	NS-Sq (n)	NS-x (n)	All-cause mortality (n)	*p*
PAPP-A	0.77 [0.57; 0.86]	48	3	19	8	5	35	0.324
	1.04 [0.98; 1.12]	48	2	24	7	8	41	
	1.54 [1.36; 1.77]	48	5	16	8	9	38	
	p_{trend}							0.322
PAPP-A2	0.18 [0.13; 0.22]	48	3	20	4	5	32	< 0.001
	0.33 [0.28; 0.37]	48	2	19	7	11	39	
	0.69 [0.57; 0.88]	48	5	20	12	6	43	
	p_{trend}							< 0.001

Number of events in low, middle and high PAPP-A or PAPP-A2 tertile groups. Median (25th percentile; 75th percentile) concentrations of PAPP-A and PAPP-A2 are shown for each tertile group. In addition, deaths among the various lung cancer subtypes are shown. Values are reported a numbers (n) of patients. P-values, log-rank test for equality of survivor function or test for trend of survivor function across ordered tertile groups (p_{trend}).

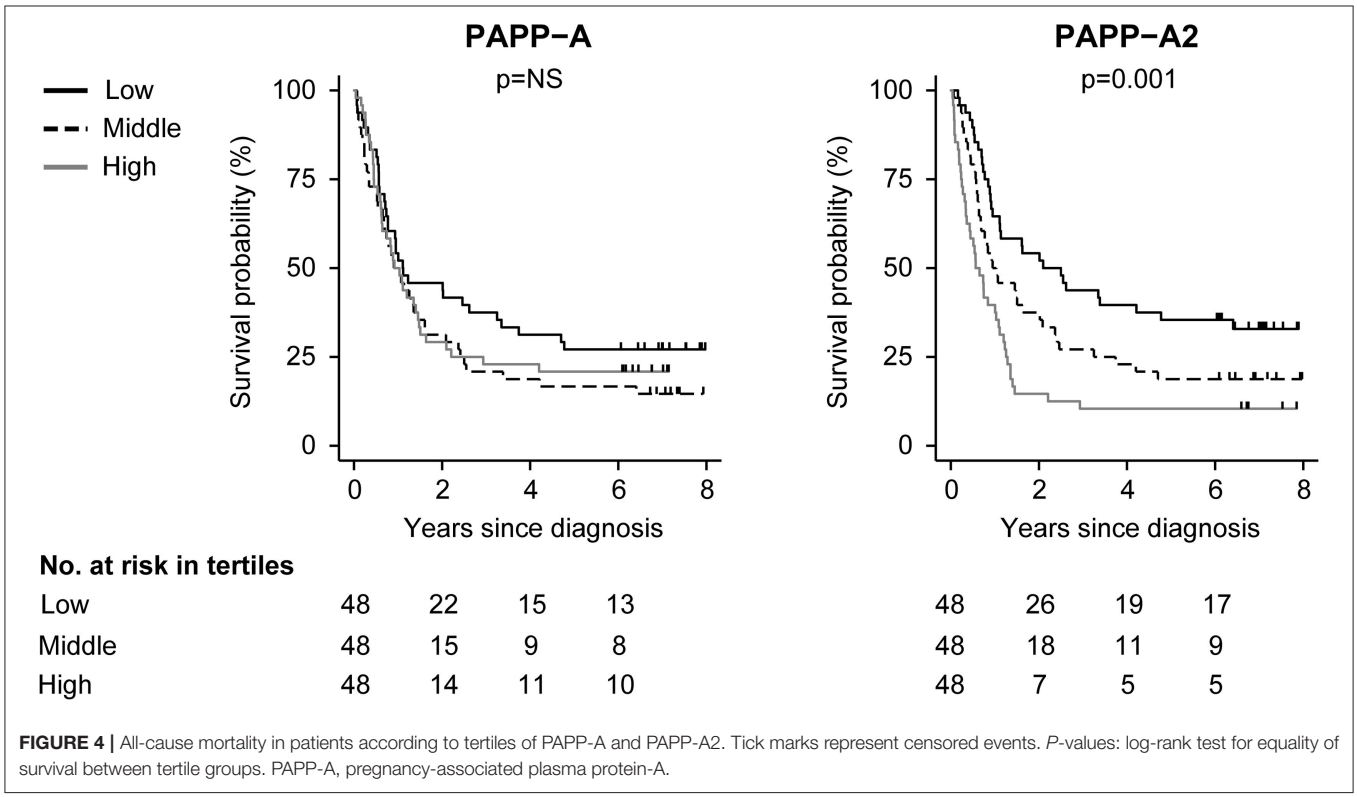

FIGURE 4 | All-cause mortality in patients according to tertiles of PAPP-A and PAPP-A2. Tick marks represent censored events. *P*-values: log-rank test for equality of survival between tertile groups. PAPP-A, pregnancy-associated plasma protein-A.

this disease. However, the novel association between PAPP-A2 and lung cancer warrants further validation in external cohorts as well as functional studies to establish a causal relationship.

Early detection and treatment of lung cancer are urgent global healthcare priorities and pose a formidable challenge. Unfortunately, early symptoms, if present, are indistinct and non-specific, and the majority of patients appear with advanced disease. Thus, novel ways to identify patients and treatment options are crucial. IGF signaling clearly plays a pivotal role in the progressive transformation of normal cells into malignant derivatives and has been shown to regulate most steps of tumor progression, including sustained cell proliferation, clonal expansion, angiogenesis, migration, invasion, and colonization of secondary sites and resistance to certain anti-cancer therapies (2). PAPP-A has emerged as an oncogene, and burgeoning evidence indicates that PAPP-A is implicated in tumor formation through the amplification of IGF actions. PAPP-A is expressed by a wide range of cells of malignant origin (31, 32), being transiently increased in some cancers and constitutively expressed by others (8, 20, 33). In murine models, PAPP-A deficiency results in a delayed occurrence of age-related fatal cancers and sporadic tumors (34, 35). In 2009, Bulut et al. found increased PAPP-A levels in serum from patients with lung cancer (19). However, we were unable to confirm this finding in the present study.

TABLE 3 | Cox regression analyses.

	Range (ng/mL)	Univariable HR (95% CI)	*p*	Multivariable HR (95% CI)	*p*
ALL-CAUSE MORTALITY					
PAPP-A					
Continuous[a]		1.13 [0.84;1.51]	0.435	1.09 [0.80;1.48]	0.574
Categorical[b,d]					
Low tertile	0.77 [0.57; 0.86]	Reference		Reference	
Middle tertile	1.04 [0.98; 1.12]	1.41 [0.89; 2.21]	0.139	1.32 [0.82; 2.10]	0.251
High tertile	1.54 [1.36; 1.77]	1.26 [0.79; 1.99]	0.328	1.16 [0.729; 1.87]	0.545
PAPP-A2					
Continuous[a]		1.30 [1.12; 1.53]	0.001	1.25 [1.05; 1.48]	0.013
Categorical[b,d]					
Low tertile	0.18 [0.13; 0.22]	Reference		Reference	
Middle tertile	0.33 [0.28; 0.37]	1.57 [0.98; 2.50]	0.060	1.51 [0.94; 2.43]	0.086
High tertile	0.69 [0.57; 0.88]	2.60 [1.64;4.14]	<0.001	2.12 [1.26; 3.58]	0.005

PAPP-A and PAPP-A2 were investigated both in univariable analyses and in multivariable analyses extended by covariables age, sex, and BMI. BMI, body mass index; CI, confidence interval; HR, hazard ratio; PAPP-A, pregnancy-associated plasma protein-A.

[a]Hazard ratio (HR) per doubling of the protein; modeled as log(marker)/log(2). Modeled using Cox proportional hazards regression.

[b]Hazard ratio with the low tertile as reference group. Modeled using Cox proportional hazards regression.

[d]For PAPP-A and PAPP-A2, low, middle and high tertile refers to the lowest, middle and highest tertiles of the protein.

Furthermore, PAPP-A was not associated with mortality and does not appear to possess potential as a seromarker in a heterogeneous cohort of lung cancer patients.

In view of the roles of PAPP-A in neoplasia, we also examined its homolog, PAPP-A2. Proteolytic activity against IGFBP-5 has been reported in various fluids and cells from several sources (4, 36–39). In mice, genetic deletion of PAPP-A2 results in normal size at birth, but there is postnatal growth retardation and bone abnormalities (23). Recently, a novel loss-of-function mutation in the human *PAPPA2* gene was discovered, resulting in a syndrome of growth retardation with elevated concentrations of IGFs, but a decreased bioactivity due to a concomitant increase in serum IGFBP-3 and−5 (14). These are the first human cases of reduced IGF-I bioavailability caused by defects in IGFBP regulation, demonstrating that PAPP-A2 has relevant consequences in human growth. Furthermore, the study confirmed the absence of functional redundancy between PAPP-A and PAPP-A2. In regard to cancer, few studies mention PAPP-A2. In 2013, a whole-exome sequencing study of lung adenocarcinoma patients identified *PAPPA2* gene mutations that were associated with prolonged survival times (40). In 2017, the present authors investigated PAPP-A, PAPP-A2 and IGF activity in pleural fluid collected at baseline from a limited number of patients with lung cancer ($n = 24$) (18). The study showed that the distribution of IGF system proteins in pleural effusions was substantially different from that of the circulating IGF system. As compared to serum, pleura contained 47-fold higher concentrations of PAPP-A and 3.3-fold higher concentrations of PAPP-A2. Although total IGF-I levels in pleura and serum were comparable, levels of free IGF-I and the ability of pleural fluid to activate the IGF-IR *in vitro* was more than 3-fold higher. These findings support that not only PAPP-A, but also PAPP-A2, modulate the IGF signaling cascade in cancers, and furthermore, indicate that the local activity of the IGF system in extravascular fluids differs substantially from that of the circulating IGF system. Finally, our previous findings support the hypothesis that PAPP-A and PAPP-A2 regulate IGF activity without affecting total IGF-I levels.

In the present study, PAPP-A2 levels were higher in patients with lung cancer than controls. Furthermore, the presence of PAPP-A2 in cancerous tissue was demonstrated by IHC and PAPP-A2 possessed prognostic ability. Further studies to test the hypothesis could lead to the establishment of PAPP-A2 as a diagnostic and prognostic biomarker. In addition, it is reasonable to assume that increased serum levels of PAPP-A2 in lung cancer patients may correlate with augmented IGF signaling in tumor cells, and thus, PAPP-A2 may also possess potential as a biomarker for IGF-I targeted therapy. Interestingly, higher PAPP-A2 levels were not unambiguously associated with advanced stages of tumor development, suggesting that the increase in PAPP-A2 may be present even at early stages. Only when assessing PAPP-A2 tertiles, an association with tumor stage was seen. By scrutinizing PAPP-A2 levels, it was clear that some patients exhibited significantly higher PAPP-A2 levels than others, and that levels differed between tumor subtypes. We speculate that the secretion of PAPP-A2 may be elevated in some tumors, whereas others do not express PAPP-A2 at a higher level than non-cancerous lung tissue. This notion is further supported by the fact that IHC staining of PAPP-A2 was only demonstrated in four out of five patients, and that staining intensity was heterogeneous and varied considerably across cell types and between patients. Of interest, staining was lacking in the one patient with SCLC. The clinically most important division is between SCLC and NSCLC, and the lack of PAPP-A2 staining supports the concept of different cancerous mechanisms in SCLC and NSCLC. IHC analysis was, however, only performed in five patients, and more patients are needed to further investigate these speculations. Nevertheless, such tumor heterogeneity has previously been shown to apply to PAPP-A.

Studies have demonstrated that some malignant cells show higher proclivity toward expressing PAPP-A than others. In women with breast cancer, overexpression of PAPP-A was observed in 79% (8), and various subtypes revealed extensive IHC staining of PAPP-A in 45 of 46 specimens (41). The *PAPPA* gene is located in a chromosomal region associated with high frequency of loss of heterozygosity in ovarian tumors (42). However, in a study of lung cancer cell lines, PAPP-A was only secreted from two out of seven (21). In mice with patient ovarian tumor grafts, PAPP-A inhibition with a neutralizing antibody showed beneficial effects only in tumors expressing moderate-to-high levels of PAPP-A (7). These findings imply that PAPP-A secretion from cancer cells contributes to growth in a tumor-specific manner, and the same may very well be the case for PAPP-A2. The growth of some malignant tumors may not be under the influence of PAPP-A2 or may only be affected by PAPP-A2 secreted from non-malignant neighboring cells or by PAPP-A2 of endocrine origin, which is present in the tumor environment. Finally, the activity of PAPP-A2 in the promotion of tumor growth is tied to the capability of cells to not only secrete functional PAPP-A2, but also express IGF-IRs. Dysregulation of signaling pathways in differentiating cells can dictate the emergence of neoplastic cells, but tumors have a diverse genetic makeup that renders them reliant on different signaling pathways for growth. Indeed, the IGF signaling pathway is only one of many, and the large number of driving forces behind different cancer subtypes is poorly understood. Collectively, inter-tumoral variability makes interpretations of the pathophysiology of PAPP-A2 difficult. Most current therapies treat cancer as a homogenous disease and customizing anti-cancer therapies to target specific neoplasms presents an ongoing challenge in the field of cancer therapy.

The increase in PAPP-A and PAPP-A2 in tumors and tumor microenvironments may also be reflective of other pathological processes. PAPP-A is often associated with inflammatory states, and levels are up-regulated by several pro-inflammatory cytokines, with IL-1β and tumor necrosis factor (TNF)-α being invariably potent promoters (43, 44). Aberrant immune responses are involved in cancer patients before clinical confirmation of disease, and chronic inflammation predisposes to and is involved in the onset of tumorigenesis (45). Furthermore, an inflammatory microenvironment has been suggested to induce proliferation of neoplastic cells (46). The mechanism as to how inflammatory signals exacerbate malignant development is poorly understood. In a cancer setting, an inflammatory milieu may potentiate PAPP-A and perhaps PAPP-A2 expression, further encouraging tumor growth. However, in this study, PAPP-A did not appear associated with markers of inflammation, and levels were not higher in NS-Sq patients, although this group displayed a higher degree of inflammation than patients with other subtypes. On the contrary, PAPP-A2 showed correlations with several markers of inflammation, and the highest levels of PAPP-A2 were found in the NS-Sq group. Thus, high PAPP-A2 levels may reflect an inflammatory state as well as cancer disease.

The IGF system and its protease system is an exciting area of research that could spur progress in cancer diagnostics and treatment, and it is conceivable that PAPP-A2 neutralizing antibodies would show beneficial effect in cancer therapy. However, most important are studies into the specific driving forces behind different subtypes and intra-tumor heterogeneity, which will facilitate a better understanding of the nature of cancer and provide insight into the development of more effective and personalized cancer therapies.

Some strengths and limitations of our study should be acknowledged to aid in data interpretation. A primary strength is the Danish nationwide health registers that offer ideal opportunities for epidemiological research. Registration of cancer cases in Denmark is mandatory and provides complete follow-up, the ascertainment of lung cancer is near complete and the free public healthcare system essentially eliminates private hospital treatment. However, this is a single center, prospective cohort study design, and although prospective studies usually have fewer potential sources of bias and confounding than retrospective studies, our results must be evaluated in light of that. Samples were collected at baseline with no measurement beyond, and thus, we were unable to evaluate dynamic changes in protein levels over time. Furthermore, the small number of cancer patients in some of the histological subgroups did not encourage profound subdivisions. The control group comprised non-cancer patients but cannot be considered healthy participants. All were referred due to suspected lung cancer but held a variety of other diagnoses. Finally, to ensure that PAPP-A and PAPP-A2 levels were analyzed in the context of lung cancer and not another undiscovered cancer disease, we excluded control patients who had an incident cancer diagnosis at another site during the first 2 years of follow-up. Eventually, our studies must be validated in a second independent cohort, and functional and mechanistic studies must be performed to establish causal relationships.

CONCLUSION

In lung cancer patients, PAPP-A2 emerged as a predictor of mortality, levels were increased as compared to controls and PAPP-A2 expression was documented in malignant tissues. PAPP-A2 may induce augmented IGF signaling in tumor cells, and further studies to test this hypothesis could lead to the establishment of PAPP-A2 as a prognostic marker or a biomarker for IGF-I targeted therapy. To confirm this association, our studies must be validated in new and external cohorts in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Central Denmark Region Committees on Biomedical Research Ethics (IRB 0005129). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RH, UE, and JF conceived and designed the study. UE, TR, and BF were responsible for patient recruitment and sample collection. RH, UE, TS, JG, and CO performed the experiments and acquired the data. Data interpretation and statistical analyses were performed by RH and UE. RH drafted the manuscript, which was revised and approved by all authors. RH had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by The Hede-Nielsen Foundation, The Danish Lung Association, The A.P. Møller Foundation for the Advancement of Medical Science, The Beckett Foundation, and Department of Clinical Medicine at Aarhus University. The funders had no influence on the design or conduct of the study.

ACKNOWLEDGMENTS

We are grateful to AnshLabs for providing PAPP-A and PAPP-A2 ELISA kits.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 05 January 2021
doi: 10.3389/fendo.2020.565557

Reduced Fragmentation of IGFBP-2 and IGFBP-3 as a Potential Mechanism for Decreased Ratio of IGF-II to IGFBPs in Cerebrospinal Fluid in Response to Repeated Intrathecal Administration of Triamcinolone Acetonide in Patients With Multiple Sclerosis

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Molecular and Structural Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 25 May 2020
Accepted: 13 November 2020
Published: 05 January 2021

Citation:
Hoeflich A, Fitzner B, Walz C, Hecker M, Tuchscherer A, Brenmoehl J and Zettl UK (2021) Reduced Fragmentation of IGFBP-2 and IGFBP-3 as a Potential Mechanism for Decreased Ratio of IGF-II to IGFBPs in Cerebrospinal Fluid in Response to Repeated Intrathecal Administration of Triamcinolone Acetonide in Patients With Multiple Sclerosis. Front. Endocrinol. 11:565557. doi: 10.3389/fendo.2020.565557

Multiple sclerosis (MS) is a chronic autoimmune disease of the brain and spinal cord causing a wide range of symptoms such as impaired walking capability, spasticity, fatigue, and pain. The insulin-like growth factor (IGF) system has regulatory functions for the induction of inflammatory pathways in experimental encephalomyelitis. We have therefore assessed expression and regulation of the IGF system on the level of IGFs and IGFBPs in serum and cerebrospinal fluid (CSF) in the course of four repeated triamcinolone acetonide (TCA) administrations in two female and four male MS patients. Sample series of 20 treatment cycles were analyzed. IGF-I and IGF-II were quantified by ELISAs, and IGFBPs were analyzed by quantitative Western ligand (qWLB) and Western immunoblotting (WIB) in order to differentiate intact and fragmented IGFBPs. The ratios of fragmented to intact IGFBP-2 and -3 were calculated in serum and CSF. Finally, the ratios of IGF-I and IGF-II to the total IGF-binding activity, quantified by qWLB, were determined as an indicator of IGF-related bioactivity. After the fourth TCA administration, the average level of IGF-I was increased in serum ($p < 0.001$). The increase of IGF-I concentrations in serum resulted in an increased ratio of IGF-I to IGFBPs in the circulation. By contrast in CSF, fragmentation of IGFBP-2 and IGFBP-3 and the ratio of IGF-II to intact IGFBPs were decreased at the fourth TCA administration ($p < 0.01$). Furthermore, reduced fragmentation of IGFBP-3 in CSF was accompanied by increased concentrations of intact IGFBP-3 ($p < 0.001$). We conclude that reduced fragmentation of IGFBPs and concomitant reduction of IGF-II to

IGFBP ratios indicate regulation of bioactivity of IGF-II in CSF during repeated intrathecal TCA administration in MS patients.

Keywords: insulin-like growth factor (IGF), insulin-like growth factor (IGF)-binding proteins (IGFBPs), cerebrospinal fluid (CSF), multiple sclerosis, IGFBP-fragment, triamcinolone acetonide

INTRODUCTION

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic progressive disease of the central nervous system that often manifests in young adulthood and particularly in women. MS is a very heterogeneous disease, with complex pathophysiology reflected by three different patterns of active white matter lesions (1). In the course of the disease, MS patients develop a variety of neurological symptoms such as depression, fatigue, paresis, and spasticity, the latter leading to severe impairment of the patients' abilities, pain, and contractures (2). Therapeutic options for MS-related spasticity include oral applications of gamma-aminobutyric acid agonists, centrally acting α2 adrenergic agonists, postsynaptic muscle relaxants, and hydrochloride salts (2, 3). Another option for MS patients with predominantly spinal cord symptoms such as spasticity is the intrathecal injection of sustained-release steroids such as triamcinolone acetonide (TCA) (4). Due to its invasive application form, repeated TCA administration is restricted to highly selected patients and applied only at experienced MS centers.

Relevance of the Insulin-Like Growth Factor System for MS

The insulin-like growth factor (IGF) system is particularly relevant in MS (5). In mice, disruption of the *Igf1* gene resulted in smaller brains, smaller amounts of oligodendrocytes, and deficits of myelination (6), whereas IGF-I transgenic mice had larger brains and increased myelin content in the brain (7). Therefore, the administration of IGF-I has been considered as an option for the treatment of MS. However, neither in rodent models nor in human MS patients, a significant effect of IGF-I alone or in combination with IGFBP-3 on demyelination or clinical impairment could be observed so far (8–11). Targeted expression of IGF-I in mouse brains in order to increase local IGF-I concentrations (12) did also not protect from experimental autoimmune encephalomyelitis (EAE). A very recent publication has demonstrated that, in fact, IGFs exert proinflammatory responses in EAE. This effect was shown to be dependent on the presence of IGF-I receptor and mediated *via* the Akt signaling pathway (13). At the cellular level, IGF-I induced the differentiation of T helper 17 (Th17) cells and therefore impacted the Th17 and regulatory T cell (Treg) balance (13), which is required for host defense and adequate immune tolerance. Notably, the Treg-Th17 balance is altered under conditions of autoimmune diseases, including MS (14, 15). Accordingly, it is essential to improve our understanding of how therapeutic approaches for MS may modulate the IGF system. The local activity of IGFs is regulated by hormone concentrations, IGF-I receptor density, and high-affinity IGF-binding proteins (IGFBPs) (5). According to the current concept of the IGF system, a proteolytic system triggers the controlled release of IGFs from IGFBPs, thereby mediating local and acute IGF effects *via* IGF-I receptors (16).

In order to assess the regulation of the IGF system in MS patients, we have quantified IGF and IGFBP concentrations in serum and CSF in response to repeated intrathecal TCA administration. We have also asked if proteolysis of IGFBPs can be postulated in serum or CSF, which may have relevance for the ratio of IGF to intact IGFBPs in both compartments.

METHODS

Patients

In our biobank, we identified paired CSF and serum samples obtained as a by-product of the administration and monitoring of TCA therapy in MS patients with spasticity. The samples were collected in the years 2009–2012 and stored at −80°C until use. The patients gave their prior consent to the use of residual clinical samples for research purposes. The ethics committee of the University Medical Center Rostock approved the use of the samples for this study (approval A 2016-0088). We included only complete sample series representing the full treatment cycle (**Figure 1**) consisting of four applications of TCA (applied every second day in a dose range of 40–80 mg). CSF was collected immediately before intrathecal TCA injection and peripheral blood after lumbar puncture. Each patient received at least one treatment cycle of intrathecal TCA injections before the collection of the samples analyzed in this study. The previous treatment cycles of the patients ended about 3 months before. We examined paired CSF and serum sample series from 20 treatment cycles (six treatment cycles of two postmenopausal female patients and 14 treatment cycles of four male patients), resulting in the analysis of a total of 80 CSF and 80 serum samples. The patients were 52.9 ± 9.5 years old (mean ± standard deviation), had a median disease duration of 10 years (range: 4–26), and were characterized by relatively severe disability (Expanded Disability Status Scale score: 6.2 ± 1.1) and a body mass index of 28.3 ± 5.1. Only one of the patients received a disease-modifying treatment (glatiramer acetate) in addition to the intrathecal TCA injections.

Analysis of Insulin-Like Growth Factors and Insulin-Like Growth Factor-Binding Proteins

IGF-I and IGF-II were studied in serum and CSF from all patients using commercial ELISAs according to the instructions

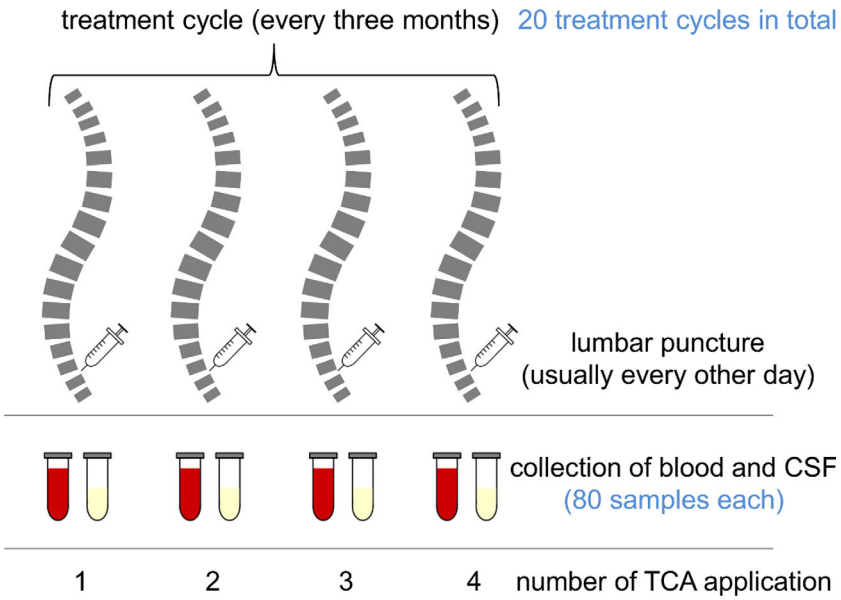

FIGURE 1 | Chart of a treatment cycle and sample collection. Multiple sclerosis (MS) patients suffering from spasticity were treated with intrathecal triamcinolone acetonide (TCA) every 3 months. Each treatment cycle consisted of four TCA applications (usually every other day). The patients were lumbar-punctured, and to avoid CSF pressure differentials, the CSF was drained and replaced with the same amount of TCA solution. After the procedure, peripheral blood was drawn for routine laboratory analyses. Remaining sample material was stored in a biobank at −80°C.

of the manufacturer (product codes: E20 and E30, Mediagnost, Reutlingen, Germany). Concentrations of IGFBP-2 and -3 were determined in CSF and serum samples using quantitative Western ligand blotting, as described previously (17). Intraassay and interassay variations were smaller than 20% for the analytes, and limits of quantification were 0.25 ng for IGFBP-2 and 1 ng for IGFBP-3. Serial dilutions of human IGFBP-2 and -3 standards were used for the quantification of IGFBP-2 and IGFBP-3. In addition, Western immunoblotting was used for the study of the structural integrity of IGFBP-2 (32 kDa) and IGFBP-3 (band doublet between 40 and 43 kDa) in CSF and serum, also as described before (18). In brief, for the identification of IGFBP-2 and IGFBP-3 after transfer to polyvinylidene difluoride membranes, blots were incubated for 2 h at room temperature with specific antibodies (goat anti-human IGFBP-2, 1:1,000, Cat. No. SC-6002, Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit anti-human IGFBP-3, 1:600, Cat. No. 13216, Cell Signaling Technology). After five consecutive washing steps for 5 min in Tris-buffered saline containing 0.05% Tween20, the membranes were incubated for 1 h with secondary antibodies (anti-goat or anti-rabbit IgG, 1:2,500). Again, recombinant human standards were used as positive controls, and molecular weight markers were used for estimating the molecular weights of IGFBP fragments. As a measure of IGFBP degradation, the ratio of fragmented to intact IGFBP was calculated for each lane. For the comparison of molar ratios, the concentrations of the IGF compounds were normalized by their molecular weight. Accordingly, IGF-I and -II levels were divided by 7.5, and IGFBP-2 and -3 levels were divided by 32 and 41.5, respectively.

Statistical Analyses

The data analysis was performed using SAS software, Version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA). Descriptive statistics and tests for normality were calculated with the UNIVARIATE procedure of Base SAS software. CSF and serum data were approximately normally distributed and were analyzed by repeated measurement analyses of variance using the MIXED procedure of SAS/STAT software. The repeated measurement ANOVA model for CSF and serum data (statistical model #1) contained the fixed factor application (levels: I, II, III, and IV) and the covariate treatment cycle. Repeated measures on the same patient (treatment cycle, application) were taken into account by the REPEATED statement of the MIXED procedure using the SUBJECT=patient option to define the blocks of the block-diagonal residual covariance matrix and the TYPE=CS option to define their covariance structure. Least-squares means (LS-means) and their standard errors (SE) were computed for each fixed effect in the model, and all pairwise differences of these LS-means were tested by the Tukey-Kramer test, a procedure for pairwise multiple comparisons. The SLICE statement of the MIXED procedure was used for performing partitioned analyses of the LS-means. With respect to the small number of patients included in the present study and the imbalanced gender contribution, gender was not included in the statistical analysis presented in **Figures 2**–**6**. Nevertheless, the effect of gender was considered by extending the above statistical model #1 by *gender* as a fixed factor in a separate statistical analysis (statistical model #2). Test results were considered significant if $p < 0.05$.

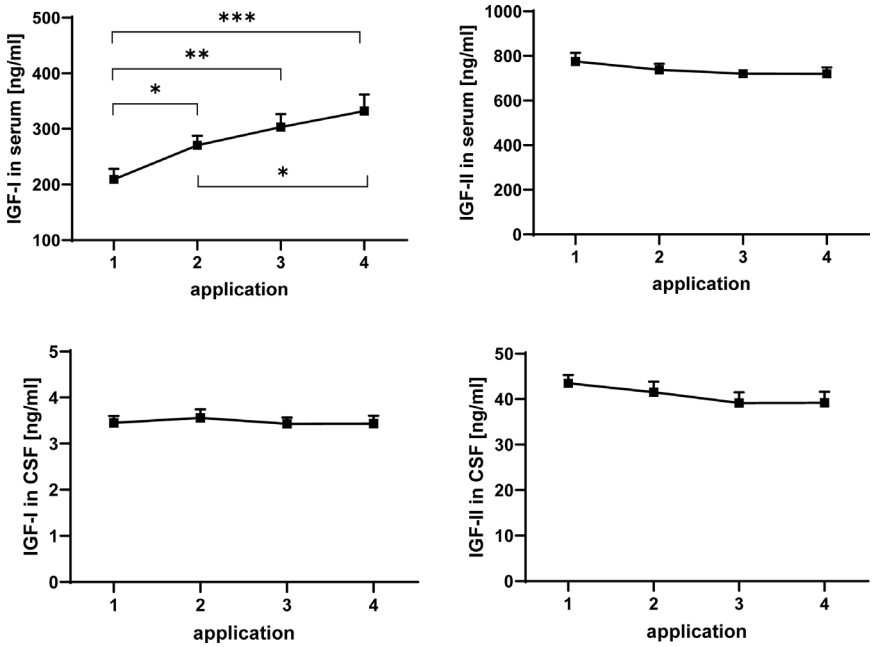

FIGURE 2 | Concentrations of IGF-I (left panels) and IGF-II (right panels) in serum (upper panels) and CSF (lower panels) in MS patients at consecutive time points of intrathecal TCA injection. The data are based on sample series from 20 treatment cycles (14 cycles in four males and 6 cycles in two females) and are given as means ± SEM (significant differences are indicated; *p < 0.05; **p < 0.01; ***p < 0.001).

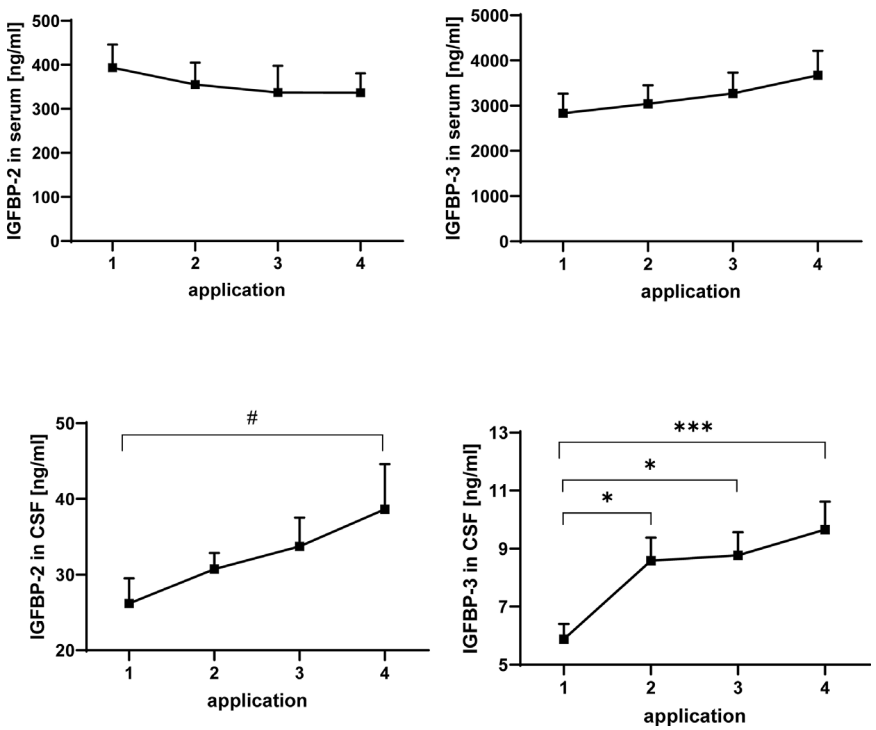

FIGURE 3 | Concentrations of IGFBP-2 (left panels) and IGFBP-3 (right panels) in serum (upper panels) and CSF (lower panels) in MS patients at the time points of intrathecal TCA injection. The data are based on sample series from 20 treatment cycles (14 cycles in four males and 6 cycles in two females) and are given as means ± SEM ($^{\#}$p < 0.067; *p < 0.05; ***p < 0.001).

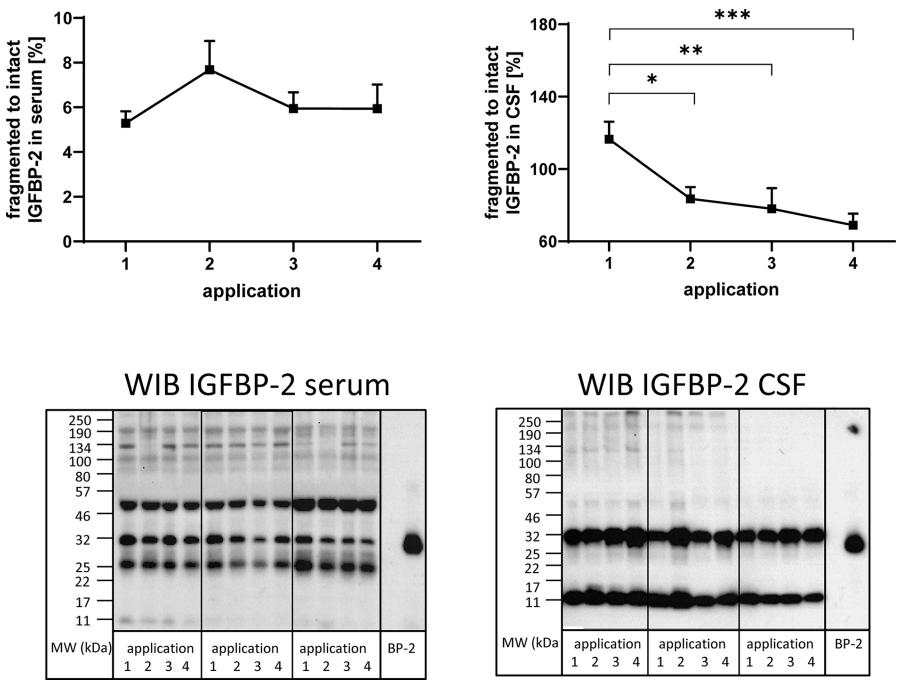

FIGURE 4 | Fragmentation of IGFBP-2 in serum (left panel) and CSF (right panel) from MS patients in the course of repeated intrathecal TCA injections. A subset of samples from 14 treatment cycles (nine cycles in four males and five cycles in two females) was considered for this analysis. Fragmentation was calculated by the average ratio of fragmented (11 kDa) *versus* intact (32 kDa) IGFBP-2 and is presented in percentages (means ± SEM; significant differences are indicated; *p < 0.05; **p < 0.01; ***p < 0.001). On the lower panels examples for the Western immuno blot (WIB) are provided for the analysis of serum and CSF. All samples from a treatment cycles I to IV were loaded on the same gel and recombinant human IGFBP-2 (BP-2) was loaded as the positive control.

RESULTS

Analysis of Insulin-Like Growth Factor-I and Insulin-Like Growth Factor-II in Serum and Cerebrospinal Fluid

The concentrations of IGF-I and -II in serum and CSF were quantified by ELISA. Repeated TCA application had a significant enhancing effect on the concentration of IGF-I in serum (**Figure 2**) when all samples were considered in the analysis (application 1 *versus* application 4: $p < 0.001$). Within the treatment cycles a progressive increase of serum IGF-I can be described, with significant increases between application #1 and #2 ($p < 0.05$) and additional increases between application #2 and #4 ($p < 0.05$). If gender was included in the statistical model (model #2), the male groups was characterized by higher concentrations of IGF-I in all serum samples ($p < 0.01$; data not shown). In CSF from the same patients, application of TCA had no effect on the IGF-I. In addition, significant effects of repeated TCA injections could not be observed on the concentrations of IGF-II in serum or CSF.

Analysis of Insulin-Like Growth Factor-Binding Proteins in Serum and Cerebrospinal Fluid by Western Ligand Blotting

IGFBPs were analyzed by Western ligand blotting in serum and CSF of TCA-treated MS patients (**Figure 3**). Similar to IGF-I, IGFBP-3 concentrations in serum were higher in males than in females (statistical model #2: $p < 0.05$, data not shown). While in serum the concentrations of IGFBP-2 and IGFBP-3 were not altered in response to intrathecal TCA injections, the average IGFBP-3 levels in CSF were elevated from TCA application #1 to TCA application #4 ($p < 0.001$). The increase of IGFBP concentrations in the CSF was evident in the sample series of both genders, even though statistical significance was reached only for IGFBP-3 within the female group ($p < 0.05$). A significant increase of IGFBP-3 in CSF was present already at the time of application #2 if compared to application #1 but not further increased to a significant extent between application #2 and #4. The increase of IGFBP-2 in response to TCA application between application #1 and #4 only reached border significance ($p < 0.067$) but was identified if the statistical model included gender as a fixed effect (statistical model #2: application #1 *versus* application #4: $p < 0.05$).

Identification of Insulin-Like Growth Factor-Binding Protein Fragments and Ratio of Fragmented Versus Intact Insulin-Like Growth Factor-Binding Proteins

In order to investigate the presence of IGFBP fragments in serum and CSF, Western immunoblotting was used. IGFBP-2-related immunoreactivity was present at four and two different molecular weights in serum and CSF (**Figure 4**), respectively. In serum, bands around 50, 32, 25, and 11 kDa were identified, while in CSF, bands

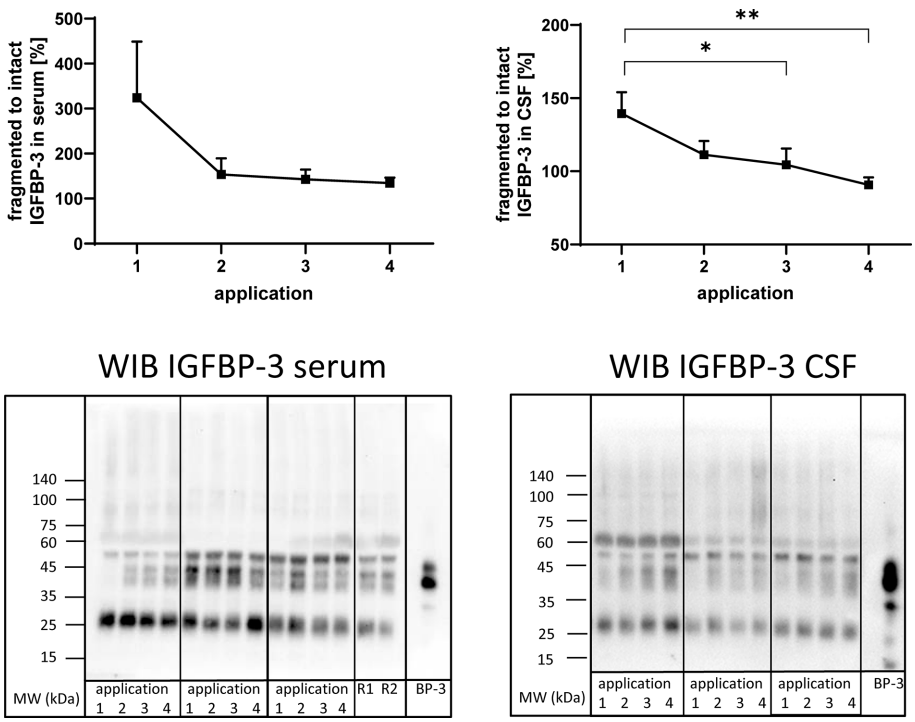

FIGURE 5 | Fragmentation of IGFBP-3 in serum (left panel) and CSF (right panel) from MS patients at the time points of intrathecal TCA application. A subset of samples from 11 treatment cycles (seven cycles in four males and four cycles in two females) was used in this analysis. Fragmentation was calculated by the average ratio of fragmented (25 kDa) *versus* intact (40–43 kDa) IGFBP-3 and is presented in percentages (means ± SEM; *$p < 0.05$; **$p < 0.01$). On the lower panels examples for the Western immuno blot (WIB) are provided for the analysis of serum and CSF. All samples from treatment cycles I to IV were loaded on the same gel and recombinant human IGFBP-3 (BP-3) was loaded as the positive control. R1 and R2 represent technical replicate samples for normalization of different experiments.

around 32 and 11 kDa were detected. The ratio of fragmented (11 kDa) to intact (32 kDa) IGFBP-2 was higher in CSF than in serum by more than one order of magnitude ($p < 0.001$). As an effect of repeated TCA administration, the ratio of fragmented (11 kDa) to intact (32 kDa) IGFBP-2 decreased in CSF (application 1 *versus* 2, 3, or 4: $p < 0.01$). The reduction of IGFBP-2 fragmentation in CSF in the course of the TCA treatment cycle was also significant when the female group was assessed separately ($p < 0.01$; data not shown). The ratio of the 25 kDa or 50 kDa IGFBP-2 band to intact IGFBP-2 was not affected by TCA application (data not shown).

Antibodies directed against IGFBP-3 detected intact IGFBP-3 (40-43 kDa) and additional bands characterized by higher (≈55 kDa) or lower (≈27 kDa) molecular weight in serum and CSF (**Figure 5**). As an effect of repeated intrathecal TCA application, the ratios of fragmented to intact IGFBP-3 decreased in CSF samples from TCA applications #3 and #4 compared to application #1 ($p < 0.05$).

The Molar Ratios of Insulin-Like Growth Factor-I and Insulin-Like Growth Factor-II to the Sum of Insulin-Like Growth Factor-Binding Proteins Quantified by Western Ligand Blot

In serum, the ratio of IGF-I to IGFBPs was significantly ($p < 0.05$) increased between application #1 and #3 (**Figure 6**). The ratio of IGF-II to the sum of IGFBP-2 and -3 (**Figure 6**) was significantly reduced in CSF after the initial TCA application ($p < 0.01$). This reduction (TCA application #1 compared to applications #3 and #4) was also significant when the samples from men and women were analyzed separately ($p < 0.05$; data not shown).

DISCUSSION

IGF-I is required for brain growth and development, and the lack of IGF-I in knockout mice resulted in reduced brain growth and hypomyelination (6). In addition, IGF-I, as an effector of Th17/Treg cell balance, induced proinflammatory responses in EAE (13). Therefore, IGF-I is not only considered as a neuroprotective agent but is also discussed concerning health-related fitness in MS patients after aerobic training (19). Accordingly, substantial reasons argue for the assessment of the IGF system in MS patients, both in CSF and in the circulation. Here, we analyzed the dynamics of IGF-I and -II and IGFBPs in serum and CSF in male and female MS patients in response to four consecutive intrathecal TCA injections. In order to estimate IGF-related bioactivity in serum and CSF, we calculated the molar ratios of IGF to the sum of intact IGFBPs detected by quantitative Western ligand blot in both compartments.

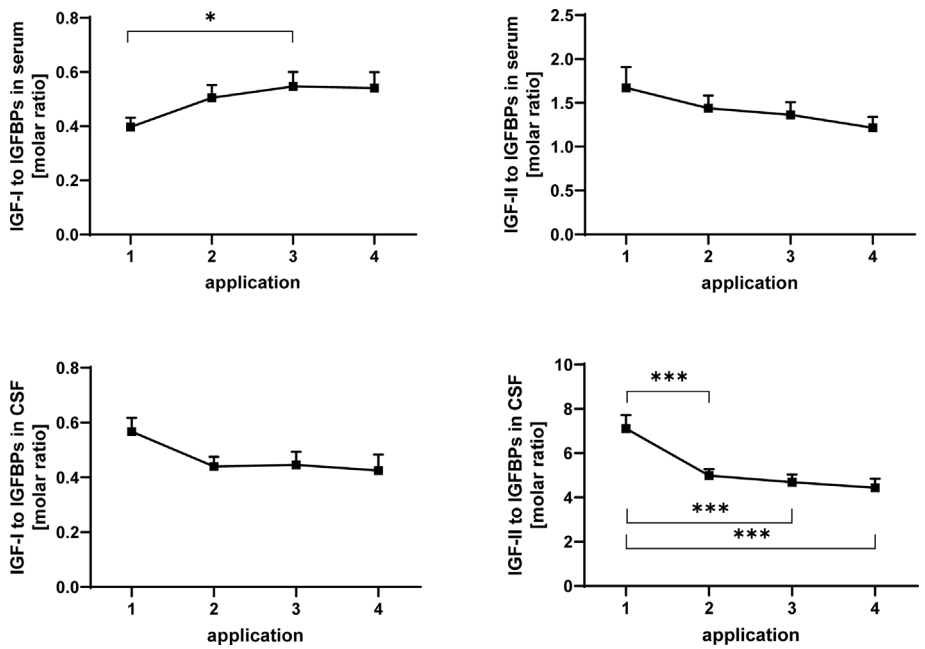

FIGURE 6 | Molar ratios of IGF-I (left panels) and IGF-II (right panels) in serum (upper panels) and CSF (lower panels) to total IGF-binding activity detected by Western ligand blotting. The samples were obtained from MS patients with spasticity receiving treatment cycles of four intrathecal TCA applications. Total IGFBP activity is composed of IGFBP-2 and IGFBP-3. The plot depicts the average change in molar ratios over 20 treatment cycles (14 cycles in four males and 6 cycles in two females). Data are given as means ± SEM (*$p < 0.05$; ***$p < 0.001$).

Compounds From the Insulin-Like Growth Factor System in Serum and Cerebrospinal Fluid From Multiple Sclerosi Patients

Our results on the concentrations of IGFs and IGFBPs in serum generally correspond to previous findings in MS patients (20, 21). Compared to other studies in MS patients (22–24), but also to published reference concentrations (25), we found higher IGF-I concentrations in the serum of our patients. The reason for this could be related to differences in severity and stage of the disease, therapeutic interventions, different age, and gender distributions, and, last but not least, to different analytical techniques. As we have identified an inducing effect of intrathecal TCA administration on serum concentrations of IGF-I, it is possible that repeated TCA injections may have long-lasting enhancing effects on serum IGF-I concentrations in MS patients.

For CSF, concentrations of IGFBP-3 have already been published in a similar range as we found in this study (20). By contrast, IGFBP-2 concentrations in the CSF are 5- to 10-fold lower in the present study compared to other studies in MS patients (20, 26). In these studies, IGFBP-2 ELISAs were used. Therefore, the strong differences could be related to the different methods used and/or to the presence of IGFBP-2 fragments in the CSF (27), which potentially were included in the measurement by quantitative ELISA but not in quantitative Western ligand blotting. Between 3 and 40 years of age, the CSF concentrations of IGFBP-3 range between 14 and 22 ng/ml (28). However, CSF IGFBP-3 levels decrease with age (28), possibly explaining why lower concentrations (5–13 ng/ml) were observed in the patients, which were up to 67 years old. The measured concentrations of CSF IGF-I (3.4–4 ng/ml) and -II (37–49 ng/ml) were higher (20) or in a similar range (23, 27) compared to published results from the literature.

Association of Age and Gender With the Insulin-Like Growth Factor System

In this study, four male and two female patients aged between 40 and 67 years were included. Due to the small number of patients, gender-specific differences could not be clearly delineated. However, according to published reference levels (25), males (at the age of 51–55 years: 119 ng/ml) have slightly higher concentrations of IGF-I in serum than females (at the age of 51–55 years: 110 ng/ml). Moreover, as an effect of age, the level of IGF-I in serum is roughly 10 ng/ml lower per decade of life (29). This may be related to growth hormone (GH) secretion, which is altered during chronic or acute illness (30) and is known to control both IGF-I and IGFBP-3 levels. Interestingly, in glial cells, sex steroids and glucocorticoids have been shown to differentially regulate expression of the IGF system (31). Unfortunately, IGF-II mRNA expression was not assessed in the cited study (31). Since TCA is detectable in the periphery after intrathecal administration (32), differential steroid effects on IGF secretion can be assumed in the periphery as well. Effects of steroids on the expression and secretion of IGF-I have been already demonstrated in animal models (33) and healthy subjects (34).

Effects of Triamcinolone Acetonide Application

Serum IGF-I concentrations increased in response to repeated TCA applications. This effect was also significant in the male subgroup. A previous study showed that after aerobic training, the serum concentrations of IGF-I in MS patients are significantly correlated with muscle strength (left and right-hand strength, quadriceps strength) and walking speed (19). Low levels of IGF-I in the circulation were further associated with fatigue and cognitive impairment in MS patients (24). The positive effects of repeated TCA injections on serum concentrations of IGF-I and on the ratio of IGF-I to IGFBPs in serum from MS patients may thus have relevance for physical and cognitive functions. A potential relation to the finding that repeated application of TCA significantly increased walking distance in MS patients (35) may be addressed in future multicentric studies comprising larger patient cohorts.

In CSF, but not in the circulation, repeated intrathecal application of TCA evoked increased concentrations of IGFBP-3 and with borderline significance also of IGFBP-2. Degradation products were seen for both IGFBP-2 and -3. The elevated intrathecal levels of IGFBP-2 and -3 in the course of TCA treatment were reflected by reduced levels of distinct IGFBP-2 and -3 fragments. Proteolytic degradation of IGFBPs in the periphery is part of the physiological growth control in human development (36–38) as well as in cancer (39).

For IGFBP-2, proteolytic fragments characterized by molecular weights of 12 and 23 kDa have been described (39). Notably, both fragments can be formed by the activity of PAPP-A (40). By contrast, the nature of IGFBP-2 related immunoreactivity around 50 kDa is less clear and further investigation is required to clarify the identity of this band, although no biomarker content appeared to be connected with this signal in this study. For IGFBP-3, which is cleaved by PAPP-A2 (16), a proteolytic fragment with molecular weight smaller than 30 kDa and bigger than 25 kDa also was describe before (18, 41). Since the concentrations of IGFBP-2 and -3 in serum were in a normal range as discussed above, we have no direct evidence to assume that the substantial amounts IGFBP-fragments are due to unspecific degradation during long-term storage in this compartment. Instead, endogenous proteases may be responsible for a high turnover of intact IGFBPs as part of physiological and conditional control of IGF-system in the circulation (16). In a recent study, PAPP-A was identified also in CSF from diabetic patients with and without diabetic polyneuropathy and in control participants (42). The study by Kallestrup et al. (42) revealed a positive correlation between the Neuropathy Rank Sum Score (NRSS) and proteolytic activity (i.e., concentration of fragmented IGFBP) in CSF. Since IGFBP proteolysis in diabetic patients is positively correlated with NRSS, reduced IGFBP proteolysis may contribute to beneficial effects of repeated TCA administration in MS patients. Subsequent studies are warranted for confirmation and closer inspection of the underlying molecular events.

In order to model the interactions between IGFs and IGFBPs, we calculated the ratios of both growth factors to the binding activity detected by Western ligand blotting. As an effect of TCA application, the ratio of IGF-II to the IGF-binding activity (intact IGFBP-2 plus intact IGFBP-3) was reduced at application #4 compared to application #1. Since the concentrations of IGF-II in CSF did not change significantly during TCA treatment, we can assume that the ratio of IGF-II to IGF-binding activity is regulated in response to intrathecal TCA administration at the level of IGFBPs and more specifically at the level of IGFBP proteolysis. From the reduced ratio of IGF-II to IGF-binding activity in CSF, it might be appropriate to assume lower IGF-bioactivity at TCA application #4. With respect to the important and novel findings by DiToro et al. (13), it is possible to conclude here that reduction of IGF-related bioactivity could block inflammatory pathways also in MS patients. In fact, a direct relation between proteolytic activity and bioactivity of IGF-I has been established in pleural fluid (18). Although in CSF from diabetic patients, elevated bioactivity of IGF-I in the presence of elevated IGFBP proteolysis could not be confirmed (42).

Our retrospective study has several limitations. Lumbar puncture is an invasive procedure that is rarely performed, e.g. to confirm the diagnosis of MS. An exception is the intrathecal administration of TCA, which can be considered in a small number of MS patients with spasticity for whom no other therapies are effective. Therefore, the number of patients that could be included in this study was limited and imbalanced in terms of sex and age distribution. Furthermore, healthy controls could not be included, and thus, it is challenging to interpret absolute hormone levels determined in MS patients. Therefore, quantitative data were discussed in comparison to published hormone concentrations in serum and CSF. Moreover, we only addressed IGFBPs detected by Western ligand blotting but did not include low-abundant IGFBPs because serum and CSF were not available in quantities sufficient for multiple ELISAs. Proteolysis of IGFBPs in serum and even more in CSF has received not much attention and we do not have specific knowledge about preclinical conditions affecting the concentrations of intact *versus* fragmented IGFBPs in either matrix, be it fresh or long-term stored. It is also unknown if the substantial reductions of IGF-II to IGFBP ratio in CSF are biologically relevant since IGF-II is in high molar excess to the IGFBPs in CSF. It would thus be necessary to study IGF-II related biological activity in CSF in future studies. Finally, future studies will also have to assess the roles of PAPP-A and PAPP-A2 for the control of IGF-related bioactivity in order to test hypotheses developed or supported by the present study.

To summarize, in response to repeated intrathecal TCA administration, the IGF system is regulated differently in serum and CSF: IGF-I concentration and the ratio of IGF-I to intact IGFBPs is elevated in serum, while IGFBP-3 and, at least in tendency, IGFBP-2 levels increase in response to TCA administration in CSF. The increase of intact IGFBP-3 concentrations is reflected by reduced levels of respective IGFBP fragments in CSF and by reductions of the IGF-II to IGF-binding activity ratios during the treatment cycles. We hypothesize that intrathecally administered TCA influences IGF-II-related bioactivity either by controlling IGFBP fragmentation in CSF and/or by controlling the molar ratio of IGF-II to IGFBPs.

Accordingly intrathecal injection of TCA differentially regulates the systemic and central IGF-system in MS patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the University Medical Center Rostock and approved the use of the samples for this study (Approval A 2016-0088). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CW generated the data. AH, AT, and BF produced the figures. AT performed the statistical analysis. All authors contributed to the article and approved the submitted version.

FUNDING

The publication of this article was funded by the Open Access Fund of the Leibniz Association and the Open Access Fund of the Leibniz Institute for Farm Animal Biology (FBN).

ACKNOWLEDGMENTS

The authors thank Luong Chau for excellent technical assistance.

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Conflict of Interest: AH is related to Ligandis UG. MH received speaking fees and travel funds from Bayer HealthCare, Biogen, Merck, Novartis, and Teva. UZ received research support as well as speaking fees and travel funds from Almirall, Bayer HealthCare, Biogen, Merck Serono, Novartis, Sanofi Genzyme, and Teva. UZ received financial support for other research activities from Almirall, Bayer HealthCare, Biogen, Merck Serono, Novartis, Sanofi Genzyme, and Teva.

However, these funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the present manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 29 January 2021
doi: 10.3389/fendo.2020.620013

Controlled Signaling—Insulin-Like Growth Factor Receptor Endocytosis and Presence at Intracellular Compartments

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Ligand-induced activation of the IGF-1 receptor triggers plasma-membrane-derived signal transduction but also triggers receptor endocytosis, which was previously thought to limit signaling. However, it is becoming ever more clear that IGF-1R endocytosis and trafficking to specific subcellular locations can define specific signaling responses that are important for key biological processes in normal cells and cancer cells. In different cell types, specific cell adhesion receptors and associated proteins can regulate IGF-1R endocytosis and trafficking. Once internalized, the IGF-1R may be recycled, degraded or translocated to the intracellular membrane compartments of the Golgi apparatus or the nucleus. The IGF-1R is present in the Golgi apparatus of migratory cancer cells where its signaling contributes to aggressive cancer behaviors including cell migration. The IGF-1R is also found in the nucleus of certain cancer cells where it can regulate gene expression. Nuclear IGF-1R is associated with poor clinical outcomes. IGF-1R signaling has also been shown to support mitochondrial biogenesis and function, and IGF-1R inhibition causes mitochondrial dysfunction. How IGF-1R intracellular trafficking and compartmentalized signaling is controlled is still unknown. This is an important area for further study, particularly in cancer.

Keywords: insulin-like growth factor 1 receptor (IGF-1R), signaling, endosomes, nucleus, Golgi

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular and Structural Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *21 October 2020*
Accepted: *02 December 2020*
Published: *29 January 2021*

Citation:
Rieger L and O'Connor R (2021) Controlled Signaling—Insulin-Like Growth Factor Receptor Endocytosis and Presence at Intracellular Compartments. Front. Endocrinol. 11:620013. doi: 10.3389/fendo.2020.620013

INTRODUCTION

Insulin-like growth factor-1 (IGF-1) stimulates essential cellular processes including proliferation, differentiation, survival and metabolism and thereby is essential for normal growth and development. Upon IGF-1 binding to the IGF-1 receptor (IGF-1R), the kinase domain becomes activated, leading to autophosphorylation of specific tyrosine residues (1–4). The subsequent recruitment and phosphorylation of Insulin-receptor-substrate (IRS-1 and IRS-2) proteins (5, 6) facilitates recruitment of PI3-Kinase and activation of the AKT-mTOR pathway (**Figure 1A**). This conserved signaling pathway regulates metabolism and transcription to promote cell survival growth or proliferation (7, 8). Activated IGF-1R may also recruit Src homology and Collagen (SHC) adaptor proteins (6, 9), and IGF-1-induced SHC phosphorylation leads to activation of RAS and the MAPK pathways that mediate mitogenic, differentiation, and migratory signals (10, 11).

IGF-1R activity can facilitate tumorigenesis, maintenance of the transformed phenotype and cancer progression (12, 13). Furthermore, IGF-1 may stimulate cancer cell migration, acquisition of epithelial-mesenchymal transformation (EMT) and chemotherapy resistance. Unsurprisingly, targeting the IGF-1R has been extensively investigated as a strategy in cancer therapy. Several kinase inhibitors and blocking monoclonal antibodies that inhibit ligand binding and signal transduction, while also triggering downregulation of the receptor have been tested (14, 15). However, the fact that these inhibitors have been largely unsuccessful in clinical trials renewed attention on how regulation of IGF-1R internalization, subcellular location and signaling are controlled in normal and cancer cells.

Although once thought that when cell surface RTKs are internalized, their signal transduction is terminated, it is now generally accepted that internalized receptors, including the IGF-1R may signal from endosomal and intracellular membrane compartments, or may also regulate gene transcription by translocating to the nucleus (16–22). However, the mechanisms of intracellular trafficking and which signals determine the subcellular localization of the IGF-1R or its compartmentalization with other signaling proteins are not known. Recent studies suggest that these events are regulated in a cell type-specific way and that cell-specific signals may influence the recruitment and activation of effector proteins (20, 22). Therefore, the cell-specific IGF-1R trafficking, compartmentalization and its subcellular location may define how cells respond to different extracellular stimuli.

Here, we review recent work on IGF-1R endocytosis, post-endocytotic trafficking and IGF-1R signaling to and from intracellular membrane compartments. We review how a non-canonical trafficking pathway *via* translocation of the receptor to internal membrane compartments and its signaling from the Golgi apparatus may contribute to its activity in cancer cells. Finally, we review the functions of IGF-1R presence in the nucleus and its effects of IGF1 signaling on mitochondrial activity.

LEAVING THE PLASMA MEMBRANE-INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR UBIQUITINATION AND ENDOCYTOSIS

Whether the IGF-1R undergoes ligand-induced endocytosis or remains on the plasma membrane is determined by the recruitment of interacting proteins (**Figure 1A**). It has been suggested that under pathological conditions like cancer, the IGF-1R associates with a range of other receptor and signaling complexes at the plasma membrane (23, 24). In particular, adhesion receptors and kinases, known to associate with the IGF-1R include E-cadherin (25), β1-Integrin (26), the discoidin domain receptor 1 (DDR1) (27), focal adhesion kinase (FAK) (28, 29), Src (30), the feline-sacroma-related kinase (FER) (31). All of these have been implicated in modulating IGF-1R stability or endocytosis to promote specific cellular responses (**Figure 1A**). However, it is unknown whether or how they might influence IGF-1R endosomal trafficking.

As with other RTKs, IGF-1R endocytosis is initiated by vesicle formation on the membrane (**Figure 1B**), and endocytosis *via* clathrin-coated-pits (CCP) is considered to be the fastest and predominant mode of internalization (23, 24, 32). The formation of CCPs requires recruitment of proteins that contain a ubiquitin-interacting motif, such as epsin, Eps15, or AP-2, to the activated receptor (23, 24, 32). Once clathrin-dependent endocytosis is saturated due to a large number of surface receptors being activated, it has been proposed that alternative endocytosis mechanisms subsequently facilitate IGF-1R internalization (33–35).

A clathrin-independent mechanism of endocytosis has been described for ligand-activated EGFR *via* micro- and macropinocytic vesicles. This involves the reorganization of the cytoskeleton and dynamic membrane ruffling (36–38). Although a similar process could be possible for IGF 1R endocytosis, it has not been demonstrated. However, clathrin independent IGF-1R endocytosis also involves the formation of lipid rafts/caveolae, which are generally described as plasma membrane invaginations. Indeed, IGF-1R has been shown to co-localize with the phosphorylated version of caveolin-1, the main component of these lipid rafts (35, 39).

Ubiquitination of the β-subunit of the IGF-1R is associated with initiation of IGF-1R endocytosis (24, 35, 40). This is dependent on IGF-1R kinase activity and requires the presence of the receptor C-terminal tail (35, 41).

Four E3 ligases have been described to either directly or indirectly interact with IGF-1R to facilitate its ubiquitination. The least studied in the context of IGF-1R is HRD1, which functions in the endoplasmic reticulum (42, 43), whereas the others, Nedd4 (40, 44), MDM2 (35, 45–47) and c-Cbl (39), are well studied (**Figure 1B**). IGF-1R ubiquitination can be observed within the first 5 min of ligand-binding. Two IGF-1R ubiquitination sites at Lys^{1138} and Lys^{1141} located within the kinase domain are believed to be the key lysine residues for ubiquitination (48). It is proposed that MDM2 recruitment to the IGF-1R occurs when low amounts of IGF-1are available, leading to IGF-1R endocytosis *via* clathrin, while high IGF-1 concentrations may initiate c-Cbl-mediated ubiquitination of the receptor followed by endocytosis using the caveolin/lipid raft route (39). This supports the idea that alternative endocytosis mechanisms are activated to internalize the IGF-1R, once clathrin-dependent endocytosis is saturated (33–35). A protein complex consisting of MDM2 and the β-arrestin protein links K63-conjugated ubiquitin polypeptide chains to the IGF-1R. This mode of ubiquitination is generally associated with cell signaling responses, DNA repair and protein trafficking (49–51) (**Figure 1B**). c-Cbl attaches K48-conjugated ubiquitin polypeptide chains to the IGF-1R, which may initiate degradation of the receptor (51) (**Figure 1B**). Thus, it is possible that depending on available IGF-1 levels, different E3 ligases are recruited to the receptor to initiate ubiquitination.

Although IGF-1R kinase activity is clearly essential for recruiting the proteins that facilitate receptor internalization and ubiquitination, it is not understood how the C-terminal

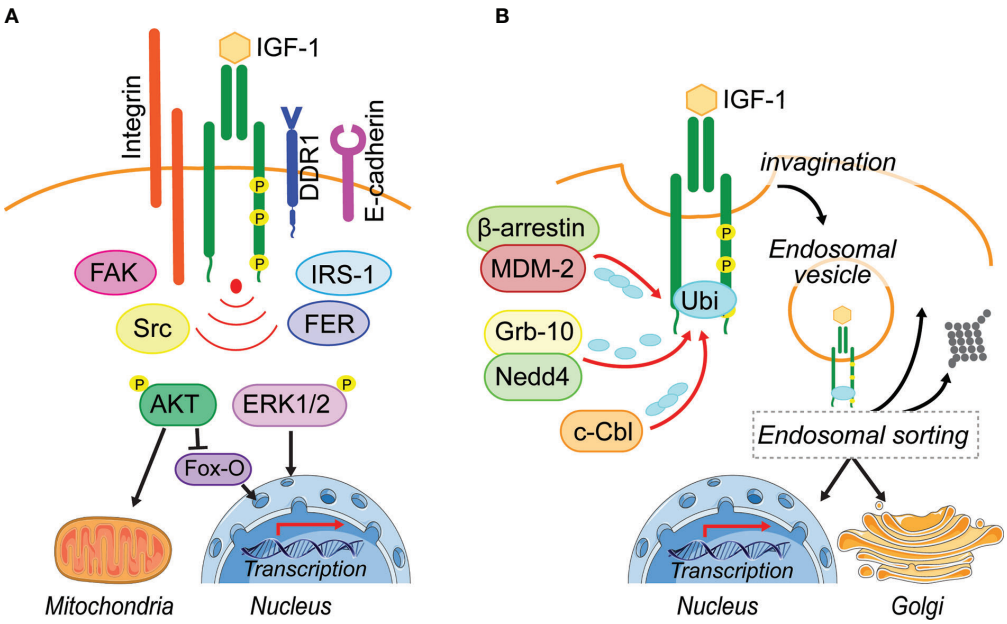

FIGURE 1 | Leaving the plasma membrane. **(A)** Located on the plasma membrane, activated IGF-1R induces two major pathways, PI3-K/AKT and MAPK/ERK1/2, to regulate cellular processes including metabolism and transcription. Different adhesion related kinases (FAK, Src, FER) and interacting proteins (IRS-1, DDR1) regulate IGF-1R endocytosis and thereby prolong or reduce IGF-1R signaling from the cell surface. In addition, these IGF-1R interacting proteins can enhance bias IGF-1R signaling or their cooperation is needed for the activation of IGF-1-induced pathways (Integrin). **(B)** Ligand-induced IGF-1R activation leads to the recruitment of E3-liages (MDM-2, Nedd4, c-Cbl) that can initiate IGF-1R poly- and mono-ubiquitination. Via membrane invagination and formation of clathrin- and caveolin-coated pits, the IGF-1R enters the cell in endosomal vesicles. It is assumed that the endosomal sorting system decides, whether IGF-1R gets degraded, travels back to the plasma membrane or translocates to intracellular membrane compartments. To this day it is unknown how the post-endocytotic IGF-1R translocation to intracellular membrane compartments, such as the Golgi and the nucleus is regulated and whether IGF-1R regulation of mitochondrial function is exclusively due to signaling transduction. Figure elements adapted from Servier Medical Art (https://smart.servier.com/), under license CC-BY3.0.

tail contributes to ubiquitin-mediated IGF-1R trafficking and degradation. Our recent study showed that IGF-1-promoted phosphorylation of the $Tyr^{1250/1251}$ site in the IGF-1R C-terminal results in enhanced IGF-1R internalization and proteosomal degradation (22). However, whether the $Tyr^{1250/1251}$ phospho-site is involved in or modulates IGF-1R ubiquitination is still unknown. The C-terminal tail contains three lysines that are putative sites for ubiquitination, but this has not been demonstrated in cells. It remains possible that phosphorylated $Tyr^{1250/1251}$ could provide a binding site for adaptor proteins or an E3 ligase that targets these sites. This would implicate the activity of domains of the receptor other than the kinase in regulating IGF-1R internalization and trafficking.

Travel Direction-Determining Insulin-Like Growth Factor 1 Receptor Trafficking Routes

CCP/caveolin-vesicles that contain internalized IGF-1R become fused with early endosomes (27, 40, 44, 52). Here the IGF-1R proteins are sorted, either targeted for degradation (24, 35), transported toward the Golgi network (22), transported to the nucleus (20, 53–56), or recycled back to the plasma membrane (57) (**Figure 1B**). Internalized ubiquitinated proteins can be detected by distinct multiprotein complexes that comprise the endosomal sorting complex required for transport (ESCRT) (58–61) and serve as signal for cargo sorting (58). The fate of internalized proteins to either undergo degradation or recycling is determined within the endosomal sorting network (61). Before membrane cargo within the early endosomes, is submitted to several rounds of cargo sorting, as the early endosome matures into a late endosome (62), cargo destined for the fast recycling route is sorted and delivered back to the cell surface (63). There is also a slow recycling route where proteins first traffic through the recycling compartments before moving back to the cell surface.

Emerging evidence indicates that cargo may also enter a retrograde trafficking route where it is transported back to the Golgi apparatus, a process that serves to maintain a robust membrane protein delivery along the Golgi-associated microtubules (18, 64–66). This particular transport route is important for β1-Integrin-promoted cell migration and adhesion (65). Although precise details of IGF-1R sorting mechanisms and which proteins are involved is still unknown, it is clear that the endosomal network is essential for selecting internalized IGF-1R and its trafficking to distinct cellular compartments. The IGF-1R also travels on a path to the Golgi apparatus as a response to IGF-1-induced phosphorylation at $Tyr^{1250/1251}$ (22). This enhances the potential for distinct intracellular signaling responses from the IGF-1R in different cells and different physiological or pathological settings.

BACK TO THE START—THE GOLGI APPARATUS AS A NEW INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR SIGNALING COMPARTMENT

The Golgi apparatus has a long-understood function in distribution, modification and secretion of newly synthesized proteins. However, it is also intimately involved in cellular processes such as cell polarization (67), directional migration (68), stress (69) and DNA repair (70). Cell migration requires coordinated communication between the plasma membrane and the Golgi apparatus (68). This may be facilitated by the retrograde trafficking of internalized plasma membrane proteins back to the Golgi apparatus (65, 71). This retrograde trafficking enables persistent cell migration because Golgi-derived microtubules act as a fast-track lane to deliver essential proteins to cell migration hot-spots, such as the sites of focal cell adhesion (**Figure 2A**) (64–66). Several key signaling proteins including Ras/MAPK (72–74) and RTKs, including MET, KIT, VGFR2, EGFR, FGFR (21, 75–77) and IGF-1R (22) have been demonstrated to locate to the Golgi apparatus, which acts as a signaling hub in normal and cancer cells (**Figure 2A**).

The rapid endocytosis and subsequent translocation of the IGF-1R to the Golgi in fibroblasts and cancer cell lines requires an adhesion-dependent autophosphorylation on $Tyr^{1250/1251}$ in the C-terminal tail (**Figure 2A**). Although evident in all cells tested, Golgi-localized IGF-1R is however a particular feature of migratory cancer cells, because cancer cell lines with low or no migratory capacity exhibit little less Golgi-localized IGF-1R. Golgi-derived IGF-1R signaling might therefore contribute to aggressive cancer cell behavior (22). In migratory cancer cell lines, IGF-1-induced SHC phosphorylation, which is required for cell migration, is dependent on an intact Golgi apparatus and also requires cell contact with the extra-cellular matrix (ECM), suggesting that the IGF-1R mediates communication between the plasma membrane and Golgi. IGF-1-induced cell migration also requires an intact Golgi apparatus (22), as well as cooperative signaling between the IGF-1R and β1-Integrin (26, 78–81) (**Figure 2A**). β1-Integrin connects the ECM with the actin cytoskeleton of cells and thereby has both a structural and signaling function in cell adhesion and migration (82, 83). This suggests, that in migrating normal and cancer cell lines β1-Integrin signaling from the plasma membrane can influence IGF-1R distribution within cells and determine its presence at the Golgi apparatus (**Figure 2A**).

While β1-Integrin is a strong candidate for determining IGF-1R translocation to and its release from the Golgi in migratory cells (**Figure 2A**), E-cadherin is a strong candidate for enhancing IGF-1R stability and plasma membrane location in low- or non-migratory cell lines. E-cadherin, which is often repressed in migratory cancer cell lines and upon EMT, especially in triple negative breast cancer cells, is readily detectable in a complex with the IGF-1R at sites of cell–cell contact in cancer cells with no or low migratory capacity (25, 84). However, in confluent migratory cancer cells (with evident high levels of cell-cell contact), and under conditions where cells are unable to migrate, the IGF-1R remains in the Golgi apparatus. Therefore, E-cadherin expression in cancer cells with no or low migratory capacity may limit IGF-1R translocation to the Golgi apparatus. Regulated and exclusive expression of cadherins and Integrins has been linked to the migratory capacity of cells during embryonic development, tumor invasion and metastasis (85–87).

Thus, it is likely that IGF-1R function in facilitating cell migration through its translocation to and signaling from the Golgi is influenced by adhesion related proteins that are expressed differently depending on cell type, which may be influenced by their hormone receptor expression, and fate, as it has already been proposed (23, 24). However, the mechanisms of this interplay between adhesion receptors and IGF-1R trafficking to and from the Golgi are still unknown. It is not known how phosphorylation and or dephosphorylation of key residues on the receptor control this and how the array of signaling proteins present at the Golgi interact.

JOURNEY TO THE CENTER OF THE CELL- INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR IN THE NUCLEUS

Several RTKs have been observed in the nucleus of cancer cells. These include EGFR family members (88–90), FGFR1 and 3 (91, 92), the IR (93, 94), VEGFR (95, 96), and IGF-1R (19, 20, 52–54, 97).

Translocation of the IGF 1R to the nucleus in cancer cells is induced by IGF-1 (20, 53, 98). Nuclear IGF-1R is more pronounced in cancer cell lines, including breast cancer, prostate cancer and sarcoma cells, compared to non-transformed cells (97). Furthermore, nuclear IGF-1R has been linked to a poor outcome for cancer patients and suggested to promote a more advanced disease stage (20, 53, 98, 99). Nuclear IGF-1R traffics from the plasma membrane (97) and the levels of IGF-1R nuclear translocation are proportional to ligand-induced kinase activation, because its translocation in cancer cells can be inhibited by xentuzumab, an IGF-1/2 neutralizing antibody, or by inhibition of IGF-1R endocytosis (20, 53, 54).

The precise mechanisms of IGF-1R import into the nucleus of normal and cancer cells are still unclear because the IGF-1R does not have a nuclear localization sequence (NLS) (53, 54) (**Figure 2B**). SUMOylation of the IGF-1R induced by IGF-1R internalization was proposed to be important (54), and IGF-1R translocation in cancer cells is facilitated by a specific subunit of dynactin p150Glued (52) (**Figure 2B**). The latter study showed that IGF-1-bound and internalized IGF-1R is transported within early endosome antigen 1 (EEA1)-positive vesicles (**Figure 2B**), it becomes positioned in the nuclear pore complex by β-importin, and is subsequently SUMOylated by RanBP2 for translocation into the nucleus (52). Suppression of any of the proteins involved in this import, leads to a significant decrease in nuclear IGF-1R. However, mutation of the SUMOylation lysine sites on IGF-1R did not abolish accumulation of IGF-1R in the nucleus (54), suggesting that additional import mechanisms exist. IGF-1R association with other proteins containing an NLS, such as IRS-1, which was previously shown to translocate to the nucleus in response to IGF-1, could also promote the

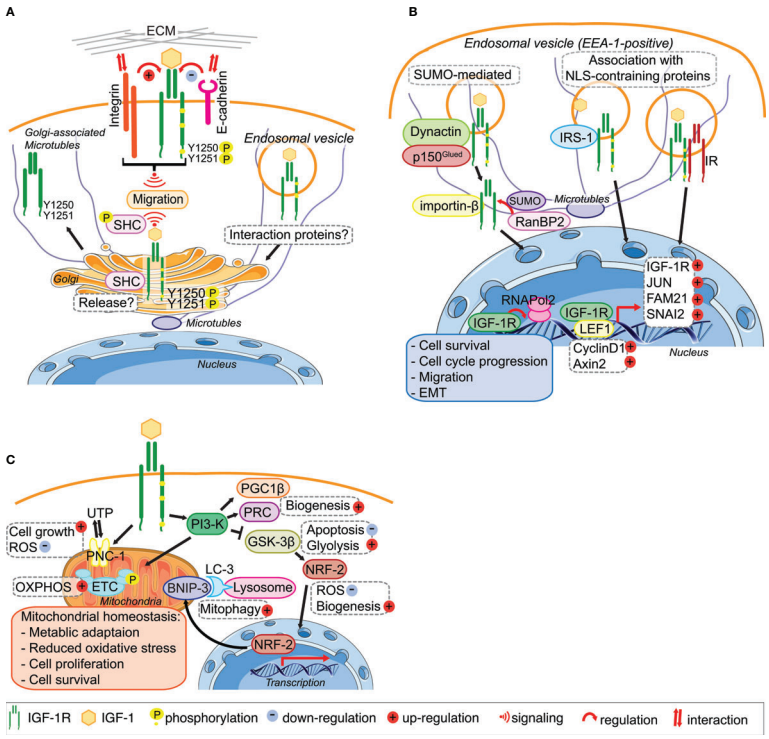

FIGURE 2 | IGF-1R trafficking routes and signaling to the mitochondria and from the Golgi and the Nucleus. **(A)** The IGF-1R translocates to the Golgi apparatus. In migratory cell lines, IGF-1R autophosphorylates $Tyr^{1250/1251}$ in an adhesion dependent manner. Phosphorylation of $Tyr^{1250/1251}$ IGF-1R leads to rapid IGF-1R endocytosis leads to activation of the MAPK pathway and results in translocation of the IGF-1R to the Golgi which promotes sustained SHC activation to facilitate migration. points. The release and retention of IGF-1R in the Golgi may be regulated by β1-Integrin and its interaction with the ECM. In cells with low or no migratory capacity, IGF-1R remains on the surface inducing signaling from the membrane. The interaction with other proteins, including E-cadherin, stabilizes the adhesion points and internalization rate of the IGF-1R is low. **(B)** IGF-1R translocates to the nucleus. IGF-1 binding to the IGF-1R induces the translocation of the membrane receptor to the nucleus. Various mechanisms have been proposed for the import of the IGF-1R to the nucleus. Nuclear IGF-1R can bind to DNA and enhance or initiate the transcription of various genes, leading to cell survival, migration, EMT and cell cycle progression. **(C)** IGF-1 signaling regulates mitochondrial function. The activation of the PI3-K pathway in response to IGF-1 induces the expression of the mitophagy regulators PGC1β and PRC. Inhibition of GSK-3 β by PI3-K activation leads to the release of NFE2L2/Nrf2, which translocates to the nucleus to enhance the expression of the mitophagy receptor BNIP-3. Activation of IGF1-R also enhances the expression of the UTP importer PNC-1, which was linked to cell growth and the reduction of ROS. Through these pathways IGF-1 signaling contributes to the maintenance of mitochondrial homeostasis. Figure elements adapted from Servier Medical Art (https://smart.servier.com/), under license CC-BY3.0.

import (100). It has also been suggested that heterodimerization with the IR, which occurs rapidly in response to Insulin stimulation (93) could promote nuclear import (55).

Nuclear IGF-1R may associate with DNA to enhance transcription (19, 54–56, 101), for example, by mediating the recruitment of RNAPol2 (20). Nuclear IGF-1R autoregulates its own expression in breast cancer cells depending on their estrogen receptor (ER) status (102) and binds the LEF1 transcription factor, which subsequently leads to upregulated cyclinD1 and axin 2 and cell proliferation (56). In HeLa cells, nuclear IGF-1R can increase the expression of SNAI2 (55), which is involved in EMT by suppressing E-cadherin expression (103). In prostate cancer cells nuclear IGF-1R facilitates expression of JUN and FAM 21, which are linked to cell survival, anchorage independent growth and cell migration, all of which are associated with advanced cancer stage (20). Nuclear IGF-1R is associated with proliferation of alveolar rhabdomyosarcoma cells (104) and contributes to chemoresistance in sarcomas and hepatocellular carcinoma (105, 106).

Overall, the results from recent studies suggest that nuclear IGF-1R facilitates an aggressive cancer phenotype. However, Aleksic et al. suggest that the sites of IGF-1R binding in DNA, and therefore the genes influenced by nuclear IGF-1R, might be cell type specific and that this could be defined by nuclear structure and chromatin organization (20). This is supported by the result of Sarfstein et al., which suggests that in presence of ER, nuclear IGF-1R cannot enhance its own expression (102).

GOING THE DISTANCE-INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR SIGNALS TO THE MITOCHONDRIA

While IGF-1 signaling in metabolism has been well studied (107), its contributions to mitochondrial function, maintenance and turnover is an emerging topic. Mitochondrial metabolism and oxidative phosphorylation (OXPHOS) provide building blocks

and energy for all cellular functions (108). At the same time, reactive oxygen species (ROS), which are a normal by-product of OXPHOS are neutralized to avoid accumulation and cell damage (109). Mitochondria synthesis (mitochondrial biogenesis) and the regulation of numbers and quality (mitophagy linked to mitochondrial fission and fusion) are well-orchestrated processes. The importance of mitochondrial quality control and mitochondrial homeostasis in the maintenance of healthy tissues is well documented (108, 110). Impaired mitophagy can lead to the accumulation of dysfunctional mitochondria and oxidative stress, which is associated with various diseases including neurodegeneration, diabetes, heart disease and cancer (108, 111–115).

IGF-1 signaling and a functional IGF-1R is essential for mitochondrial biogenesis through inducing the transcriptional mediators Peroxisome proliferator-activated receptor gamma coactivator 1 β (PGC1β) and PGC-1-related coactivator (PRC) (116, 117) (**Figure 2C**). Suppression of the IGF-1Ror the PI3-Kpathway using the IGF-1R kinase inhibitor BMS-754807 or LY294002, respectively, leads to a reduction in mitochondrial mass and biogenesis (116). IGF-1 also induces the mitophagy receptor BNIP-3 (116) through GSK-3β mediated activation of NFE2L2/Nrf2 (118) (**Figure 2C**). This highly conserved signaling pathway is conserved from *C. elegans* where it coordinates mitochondrial biogenesis with mitophagy and thereby controls cellular metabolism that is ultimately linked with lifespan (119, 120). In mammalian cells (normal or transformed), IGF-1-mediated regulation of mitochondrial biogenesis and mitophagy is more complex that in *C. elegans*. In metazoans, it needs to be integrated with metabolic status and IGF-1-stimulated mTORC1 actions in suppressing cellular macro-autophagy (121, 122). Although IGF-1 signaling may be critical for both mitochondrial biogenesis and basal mitophagy, it is not however easy to distinguish specific signals for mitophagy from general autophagy. Moreover, IGF-1 signals may control basal mitochondria health and the triggering of mitophagy in very specific cellular contexts such as cell division or differentiation.

Further evidence for an essential IGF-1 signal in maintaining healthy mitochondria comes from the IGF-1-inducible mitochondrial UTP importer, pyrimidine nucleotide carrier 1 (SLC25A33/PNC1) that is required for maintaining mitochondrial RNA and DNA (123, 124) (**Figure 2A**). Suppression of PNC-1 results in cellular accumulation of ROS under normal oxygen conditions, an increase in glycolysis and a profound induction of EMT in cancer cells (124).

Overall, it will be important to establish how IGF-1 signals and IGF-1R activity support mitochondrial function in normal cells and in phenotypically distinct cancer cells, and whether an essential component of these signals is to maintain a healthy pool of mitochondria that would prevent cancer aggressiveness that is associated with hypoxia, mitochondria dysfunction and an accumulation of cellular ROS.

Where to go From Here?—Remaining Questions in the Field

This review summarizes current knowledge on IGF-1R trafficking and signaling to and from intracellular compartments. Overall, the potential for intracellular IGF-1R signaling adds complexity to understanding and modulating IGF-1 actions in physiological and patho-physiological conditions. For example, efforts to inhibit IGF-1R signaling at the plasma membrane are not very effective, as is evident from the poor success of mAb in targeting the IGF-1R in cancer. One explanation for this is that continued signaling from intracellular pools of IGF-1R in association with specific organelles or protein signaling complexes may circumvent plasma membrane targeting. Correlating IGF-1R location and activity at the Golgi or in the nucleus with a specific subset of cancer may be a valuable biomarker for targeting IGF-1R in cancer (125). Therefore, if IGF-1R trafficking to and signaling from intracellular compartments determines its activity in cancer and contributes to an aggressive cancer behavior (20, 22), it is now important to identify the molecular regulators of IGF-1R trafficking. The functions of these proteins in selecting incoming receptors and regulating their cellular distribution and localization may the key to cellular signaling responses. Illuminating the mechanisms of IGF-1R trafficking and endosomal sorting would provide new insights on IGF signaling in normal cells and cancer cells, and may also identify potential co-targets for pharmacological intervention in cancer. Targeted therapy against proteins facilitating IGF-1R location and activity in the Golgi or the nucleus, or enhancing IGF-1R sorting toward proteosomal degradation may be beneficial in certain subtypes of cancer. Moreover, the presence of the IGF-1R at the Golgi may have potential to identify cancer subtypes where membrane targeting would not be effective. Our data on IGF-1R derived Golgi signaling also suggest that removing the receptor is important to suppress IGF-1 signaling. However, it is not yet clear whether specific antibodies that promote IGF-1R internalization could be used to direct it to the degradation machinery. It may be necessary to identify the key regulators of receptor trafficking to achieve selectivity here. 125.

AUTHOR CONTRIBUTIONS

LR and RO'C contributed to the writing of the article. All authors contributed to the article and approved the submitted version.

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