



Published in final edited form as:

J Intern Med. 2017 January ; 281(1): 25–40. doi:10.1111/joim.12528.

Targeting orphan G protein coupled receptors for the treatment of diabetes and its complications: C-peptide and GPR146

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Abstract

G protein-coupled receptors (GPCRs) are the most abundant receptor family encoded by the human genome and are the targets of a high percentage of drugs currently in use or in clinical trials for the treatment of diseases such as diabetes and its associated complications. Thus orphan GPCRs, for which the ligand is unknown, represent an important untapped source of therapeutic potential for the treatment of many diseases. We have identified the previously orphan GPCR, GPR146, as the putative receptor of proinsulin C-peptide, which may prove to be an effective treatment for diabetes-associated complications. For example, we have found a potential role of C-peptide and GPR146 in regulating the function of the retinal pigment epithelium, a monolayer of cells in the retina that serves as part of the blood–retinal barrier and is disrupted in diabetic macular edema. However, C-peptide signaling in this cell type appears to depend at least in part on extracellular glucose concentration and its interaction with insulin. In this review, we discuss the therapeutic potential of orphan GPCRs with a special focus on C-peptide and GPR146, including past and current strategies used to ‘deorphanize’ this diverse family of receptors, past successes, and the inherent difficulties of this process.

Keywords

C-peptide; diabetes; diabetes-associated complications; GPCR; GPR146; orphan GPCR

Orphan G protein-coupled receptors: an untapped source of therapeutic potential

G protein-coupled receptors (GPCRs) represent the largest receptor family encoded by the human genome. Although all GPCRs share core structural features, including seven membrane-spanning domains and conserved residues in the C and N termini, this receptor family is remarkably diverse, particularly with respect to binding partners and functions [1, 2]. Known ligands of GPCRs include peptides, fatty acids, amino acids, steroids,

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Conflict of interest statement

The authors do not have any conflicts of interest to declare.

nucleotides, and light, and activation of GPCRs by these ligands leads to a plethora of downstream signaling events initiated by stimulation of the associated G protein(s) subunits. Canonical GPCR signaling includes substitution of a guanosine diphosphate (GDP) for a guanosine triphosphate (GTP) by the alpha G protein subunit, resulting in the dissociation of the alpha subunit from the beta and gamma subunits and from the GPCR. This dissociation allows the alpha subunit to activate secondary signaling messengers, such as adenylyl cyclase, as is the case with $G\alpha_s$ ('stimulatory' G alpha protein) (Fig. 1). G alpha proteins possess intrinsic GTPase activity, and hydrolysis of the associated GTP back to GDP inactivates the G protein, wherein it can then re-associate with the beta and gamma subunits and the GPCR.

These classical GPCR signaling mechanisms have been well studied and established for many years. However, it has been increasingly clear that GPCR signaling biology is remarkably more complex than originally thought. For instance, in addition to signaling through the G alpha subunit, the beta and gamma subunits are capable of activating independent signaling cascades [3, 4]. Additionally, beta arrestin, which was traditionally thought of as a scaffolding protein involved in desensitization of GPCRs, is now known to initiate signaling through the MAP kinase pathway [2, 5–7]. It also appears that GPCRs can be quite promiscuous in terms of their binding partners. While it was once thought that one receptor (or family of receptors) responded to only one ligand (or ligand family) (i.e. the so-called 'one ligand, one receptor' hypothesis), there have now been several reports of GPCRs that bind to multiple, unrelated ligands as a result of the association between the receptor and an adapter protein, such as a receptor activity-modifying protein (RAMP) [8]. Probably the most studied GPCR that interacts with RAMPs is the calcitonin receptor-like receptor (CLR) [9–13]. The CLR can be activated by several ligands, including calcitonin, alpha- and beta-calcitonin gene-related product (CGRP), amylin, adrenomedullin, and intermedin, depending upon its interaction with RAMPs 1, 2, or 3. GPCRs have been shown to interact with other types of membrane proteins, including integrins and other types of receptors [14]. In particular, GPCRs have been shown to transactivate tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR) by activation of multiple GPCRs, including thrombin receptors, via ligand-dependent (involving matrix metalloproteinases) and ligand-independent mechanisms [15]. Furthermore, GPCRs can form homodimers or heterodimers with other GPCRs, which can have functional implications for receptor specificity and signal transduction [16]. In addition to ligand membrane-binding partner promiscuity, GPCRs are also known to be promiscuous in terms of signal transduction pathways. GPCRs can interact with multiple G protein alpha subunits (e.g. $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$) depending on the external and internal cellular milieu, and exhibit 'biased agonism' (i.e. functional selectivity) by which the GPCR ligand directs selectivity toward a specific signaling pathway [17–21]. While these complexities confer remarkable flexibility on GPCR-based signaling systems, they also present difficulties for studying this diverse family of receptors, particularly when the endogenous ligand is unknown.

GPCRs are categorized into five classes based on homology, although it has been suggested that these receptors are more appropriately classified based on ligand type [22]. Regardless of classification methodology, all groups of GPCRs include a subgroup referred to as 'orphans,' which are GPCRs that have been identified using bioinformatics or molecular

cloning but as yet do not have a known endogenous ligand. Although there is some contention over the exact number of orphan GPCRs, according to the International Union of Basic and Clinical Pharmacology (IUPHAR) there are currently over 70 orphan GPCRs with unknown function or ligand [22]. Considering that 30–50% of drugs currently on the market in the USA target GPCRs [23], orphan GPCRs represent a significant untapped source of therapeutic potential for the treatment of diseases such as diabetes and its associated complications. As a testament to this assertion, several novel therapeutics have been developed in recent years that target orphan GPCRs or recently ‘deorphanized’ GPCRs to treat diabetes and its associated complications.

GPCRs, orphan GPCRs, and diabetes (Table 1)

Traditionally, patients with diabetes have been treated with injectable insulin or oral agents. Although insulin therapy has been the mainstay treatment of type 1 diabetes as it lowers blood glucose levels, it must be carefully administered in order to approximate physiological insulin delivery. Though many formulations exist (regular, intermediate-, and long-acting insulin, human and animal origins, etc.), the use of these products requires substantial empirical adjustment. Significant side effects limiting their therapeutic effectiveness can include hypoglycemia, allergic reactions, and antibody-mediated insulin resistance. Oral agents are typically the first-line therapy in patients with type 2 diabetes. These act by either stimulating insulin secretion from the beta cells of the pancreas (sulfonylureas such as glipizide or gliburide), or increasing insulin sensitivity, enhancing glucose uptake, and decreasing gluconeogenesis in peripheral tissues (biguanides). These approaches are not without their complications, however, and can include side effects such as hypoglycemia, abdominal pain, nausea, or diarrhea stemming from their effects on either the endocrine or peripheral tissues.

Targeting GPCRs for the treatment of diabetes and its associated complications poses some definite advantages. In many cases, therapies targeting GPCRs require only once or twice daily dosage regimens, providing a significant advantage for patient compliance. Monotherapy with these agents may have some utility but GPCR-based therapeutics may have a more substantial role as combination therapies with traditional oral agents. Current GPCR-targeting therapies include glucagon-like peptide 1 (GLP-1)-based agonists, the related DPP4 antagonists (both reviewed in [24]), bromocriptine, and serotonin agonists. More recently GPCR40 agonists and selective endothelin-A receptor antagonists have received interest.

The GLP-1 agonists and DPP4 inhibitors are the most well developed of the therapies for diabetes based on GPCRs. GLP-1 is an incretin hormone that is released from the gut after a meal and interacts through a GPCR, GLP1R. GLP-1 stimulates insulin secretion, islet proliferation, and cytoprotection while inhibiting glucagon secretion [25, 26]. It was suggested as a therapeutic target in type 2 diabetes in 1992 [27] and the first pharmaceutical agent based on GLP-1 came to market in 2005. GLP-1 has a short half-life of usually 1–2 min that is the result of inactivation by the DPP4 enzyme [28]. Several pharmaceutical strategies have aimed to remove this limitation. A GLP-1-mimetic from the Gila monster called exendin-4 has close to 50% homology and binds the receptor with the same affinity as

human GLP-1 [29]. It is resistant to DPP4 and has a half-life of 30 min or 2–3 h depending on the route of administration (intravenous or subcutaneous, respectively). Synthetic analogs (exenatide) have now been developed, and in combination with metformin and sulphonylureas have been shown to lower body weight and HbA1c [30–32]. Alternatively the half-life of the native GLP-1 has been extended through structural modifications, such as amino acid substitutions and C16 fatty acid attachments, to promote binding to serum albumin (e.g., liraglutide) and to produce resistance to DPP4 activity and decreased clearance [33].

Inhibition of DPP4 serves as a therapeutic target, as it can also prolong endogenous GLP-1 concentrations that are typically observed after a meal, and increase fasting levels of GLP-1. Several DPP4 inhibitors have been approved by the FDA including algoliptin (xanthine-based compound), saxagliptin (substrate-like inhibitor), and sitagliptin (non-substrate-like inhibitor) [34–36]. However, both DPP4- and GLP-1-based therapies have been at the center of an academic debate [37–40] over the risk of pancreatitis and pancreatic cancer associated with incretin-based therapies. In 2013, the American Diabetes Association and The Endocrine Society called for efforts to investigate these potential complications [41, 42], though currently European and US federal agencies have not altered recommendations on the use of these agents.

Another potentially promising previously orphan GPCR that is being pursued as a therapeutic target is GPR40. GPR40 is expressed in pancreatic beta cells and is activated by medium- or long-chained free fatty acids to stimulate insulin secretion in a glucose-dependent manner (for reviews see [24, 43]). The secretion of insulin in response to high glucose occurs through two phases: (i) exocytosis of a pool of insulin granules already at the plasma membrane; and (ii) mobilization of intracellular granules to the plasma membrane. This second phase is potentiated by free fatty acids [44]. Half of the activity of free fatty acids can be attributed to GPR40 with the rest being the result of intracellular metabolism to long-chain coenzyme A esters [45, 46]. There is some debate regarding whether GPR40 has any impact on intracellular fuel metabolism [47, 48], probably due to differences in experimental models; nevertheless, it appears to act via a mechanism that is at least partially independent from intracellular fatty acid metabolism. In addition to its role in insulin secretion and hypoglycemic events, GPR40 has also been linked to incretin responses to free fatty acids [49]. A loss of function mutation in the *GPR40* gene is found in 0.75% of healthy subjects and is associated with obesity and impaired insulin secretion [50]. A large number of GPR40 agonists are currently being developed. It has become clear in recent years that biased agonism among several GPCRs represents an important design consideration in the development of agonists. Different agonists may bias a receptor to certain activation states that result in the activation of specific signaling pathways and therefore potentially different biological outcomes [20]. This is particularly pertinent to GPR40 which has been coupled to $G\alpha_s$ and $G\alpha_q$ signaling pathways [51] and has been proposed to also activate $G\alpha_i$ in some circumstances [52]. Furthermore in the case of GPR40, as well as other GPCRs, alternative (allosteric) ligand-binding sites, even for the same agonist, exist and can have distinct or modulating effects on the receptor [53]. This has been demonstrated in the case of the most well-developed GPR40-based clinical candidate to date, fasilifam (TAK-875; Takeda Pharmaceutical Company) [54]. Fasilifam is an oral agent that enhances glucose-dependent

insulin secretion in rat models and improves hyperglycemia [55, 56]. Importantly, there is a low risk of hypoglycemia in these models and no evidence of beta cell toxicity. In a human Phase II clinical trial, monotherapy reduced HbA1c significantly [57]. The Phase III clinical trial of Tak-875 was voluntarily terminated however due to concerns related to liver safety [56]. Considering that GPR40 has not been identified in the liver, it is likely not a direct GPR40 effect. Other agonists are being developed that target different allosteric binding sites on GPR40 in order to take advantage of the incretin-stimulating effects of GPR40 [58, 59].

GPR40 antagonists may eventually also play a role in long-term diabetes management. GPR40 null mice have some degree of protection of beta cells during high-fat diet-induced insulin resistance [60]. Pharmaceutical agents designed to exert antagonistic effects have been reported to reduce beta cell loss but do not impact hyperglycemia [61, 62]. Overall however this type of strategy may help preserve beta cell function in subjects with type 2 diabetes in the long term or serve as a protective strategy in pre-diabetic individuals.

The D2 dopamine receptor is also a GPCR and bromocriptine, a sympatholytic that targets this receptor, has recently been approved for the treatment of type 2 diabetes (reviewed in [63]). It is thought that low hypothalamic dopamine levels and excessive sympathetic tone in the CNS alter the metabolic state resembling changes in mammalian species that undergo hibernation. Such animals switch to a state of insulin resistance in muscle and liver along with accelerated hepatic glucose production and gluconeogenesis, hyperglycemia, adipocyte insulin resistance, increased lipolysis, enhanced fat oxidation with increased plasma free fatty acids and triglycerides as well as obesity [64–67]. Phase II and III clinical trials have shown improvement in levels of HbA1c, plasma triglycerides, and free fatty acids when given either as monotherapy or combination therapies with oral hypoglycemic agents [68–70]. There is also evidence from cardiovascular safety trials that bromocriptine (Cycloset) may improve major adverse cardiovascular outcomes (myocardial infarction, stroke, and death) in patients with type 2 diabetes [70].

Despite these advances in treating diabetes and its complications by targeting GPCRs and orphan GPCRs, diabetes-associated microvascular dysfunction remains an immense clinical challenge. More studies are needed to evaluate the therapeutic potential of additional GPCRs and orphan GPCRs to treat this devastating group of complications. However, the development of novel therapeutic agents based on orphan GPCRs depends heavily on the ability to identify their endogenous ligands, or to deorphanize them.

Deorphanization strategies: past, present, and future

Classically, identification of ligand–receptor pairs depended upon purification of biologically active compounds from large quantities of tissue extracts, which were used to assess activity of cloned receptors overexpressed in null cell lines. With the advent of the Human Genome Project and the formation of readily accessible databases containing the genome sequences of humans and many other species, targeted drug development, or ‘reverse pharmacology’ became possible (Fig. 2) [71, 72]. Using this strategy, a potential drug target is identified based on sequence homology or gene expression, and synthetic ligands replace purified tissue extracts used in traditional or ‘forward pharmacology’. In

recent years, reverse pharmacology has been used by the pharmaceutical industry to develop synthetic substances based on natural molecules to treat a variety of conditions. Most recently, companies have turned to computer modeling systems to predict the molecular activity of a drug at a particular receptor, which has allowed more focused *in vitro* experimentation.

Both forward and reverse pharmacology depend upon high-throughput assays, which have been a mainstay of receptor deorphanization for many years. Robotic screens, in which hundreds of receptors are overexpressed with reporter molecules (e.g. SRE-luciferase reporter proteins) in separate cell populations are exposed to potential ligands or drugs, are in use by several pharmaceutical companies. Although undirected, this strategy has had some success in identifying ligand–receptor pairs, particularly when used in combination with reverse pharmacology and PCR-based homology screening technologies. For example, the orexins were identified based on the ability of orexin-containing tissue extracts to stimulate calcium release when applied to separate cell cultures expressing over 50 different GPCRs [71, 72]. The orexins and their receptors are now known to play important roles in sleep and arousal, as well as in metabolism, and orexin receptor-based drugs are currently in development for the treatment of narcolepsy [73–75]. Other examples include ghrelin, which is produced in the gut and stimulates appetite and was found to bind the growth hormone secretagogue receptor (GHSR) [76, 77], and the adipokine apelin, which binds the previously orphan receptor APJ and plays important roles in glucose homeostasis as well as exerting cardiac and vascular effects [78].

In spite of these successes, many orphan GPCRs remain. Their deorphanization may be hindered by multiple factors, including technical difficulties inherent in high-throughput assays. For example, the downstream signaling machinery for most orphan receptors is unknown, and thus overexpression in non-native cell lines with engineered reporter proteins could prove detrimental to the activation of these receptors; their use in high-throughput assays could produce habitual false-negative results. Furthermore, some GPCRs require the presence of chaperone proteins for proper folding and trafficking to the cell membrane. Such chaperone proteins could be missing from high-throughput screening cell systems. Lastly, and most importantly, as discussed above, GPCR biology is remarkably more complex than originally realized, and thus any cell system lacking adapter proteins (RAMPs, integrins, etc), accessory receptors (e.g. additional GPCRs with which a receptor forms heterodimers), or intracellular scaffolding proteins necessary for proper signaling would render the affected orphan GPCR inactive in such a system. For these reasons, we have developed a directed ligand–receptor deorphanization strategy that is not dependent upon overexpression of the receptor in a non-native cell system but rather focuses on the pharmacological activity of the receptor in cells in which it is endogenously expressed. Thus far we have utilized this strategy to identify candidate receptors for three ligands [79–81], including a peptide hormone with tremendous potential for the treatment of diabetes-associated complications, proinsulin connecting peptide (C-peptide).

C-peptide and the deductive ligand–receptor matching strategy

Over the course of the last two decades, proinsulin C-peptide has gained recognition as a potential therapeutic target for the treatment of diabetes-associated microvascular dysfunction [82–85]. Although originally thought to be merely a byproduct of insulin prohormone processing, C-peptide is now known to possess biological activities, particularly in the microvessels, and has been shown to exert anti-inflammatory, anti-oxidant, and metabolic actions [83–86]. Additionally, C-peptide may act synergistically with insulin to maintain the vascular endothelium, although the mechanisms underlying this potential interaction have not been fully elucidated [87, 88]. However, in order to fully understand the role of C-peptide in normal physiology and, importantly, in order to develop C-peptide-based therapies, it is essential to identify the C-peptide receptor(s).

Identification of the C-peptide receptor has proven to be a difficult task. Several attempts to purify a membrane-bound receptor, with the use of affinity purification assays and molecular cloning, were unsuccessful. This difficulty could be due to the unique binding dynamics of C-peptide, as radioligand binding experiments revealed that C-peptide binds to human cell membranes likely through an interaction with a receptor complex or signalosome [89–91], which could include a GPCR coupled to an adapter protein, an integrin, a RAMP, or another type of receptor. The absence of these potential interacting proteins in non-native expression systems (i.e. C-peptide receptor ‘null’ cells) could complicate the isolation of the C-peptide receptor(s). Likewise, because the actions of C-peptide appear to be intimately linked with those of insulin [84, 88], C-peptide may exhibit cooperative binding with insulin, and thus the efficient binding and purification of a C-peptide receptor would require the presence of insulin. Therefore, isolation of the C-peptide receptor based on its biochemical and binding properties may not be feasible.

In order to identify a C-peptide receptor, we utilized a deductive ligand–receptor matching strategy developed in our laboratory to determine the receptor for the peptide hormone neuronostatin (Fig. 3) [80, 81]. Instead of depending upon the physical interaction between C-peptide and its receptor or receptor complex, this approach takes advantage of the known pharmacological effects of a ligand *in vitro* to identify receptor candidates. Briefly, cell lines or tissues known to respond to the peptide of interest (e.g. with changes in cFos mRNA expression or activation of protein kinase A) are screened for the expression of all known orphan GPCRs, as catalogued by IUPHAR [22]. Only orphan GPCRs that are expressed by all responsive cells and/or tissues, of which there are ideally three or more, are considered for further analysis. Next these receptors are subjected to bioinformatics analyses to identify homology with receptors with known ligands and potential expression profiles, which are compared to expected function and distribution based on known ligand actions. These steps are used to generate a short list of candidate receptors that are then knocked down individually using siRNA in a responsive cell line. If knockdown of a candidate receptor disrupts the normal signaling initiated by the ligand of interest, then that receptor is considered the best candidate receptor for the ligand, and is further analyzed using standard biochemical tests, including fluorescence co-localization, co-immunoprecipitation, and radioligand binding analyses.

Using this method, we identified GPR146 as the best candidate receptor for proinsulin C-peptide, as knockdown of GPR146 in the human gastric tumor cell line KATOIII resulted in a loss of C-peptide-induced cFos mRNA expression [80]. We further demonstrated that C-peptide and GPR146 co-localized on KATOIII cell membranes (Fig. 4) [80], and that pre-incubation with a neutralizing antibody against GPR146 prevented C-peptide-induced inhibition of ATP release from human erythrocytes [88]. These data strongly suggest that GPR146 is a member of the C-peptide signalosome, perhaps acting as a C-peptide receptor. However, there are multiple limitations to this deorphanization strategy. First, using this strategy assumes that the receptor is a GPCR. While multiple lines of evidence suggest C-peptide interacts with a GPCR to exert its cellular effects [84, 85], other types of membrane receptors, particularly integrins, which have been shown to activate G proteins in some instances [92], could mimic the effects of GPCRs. Secondly, our system excludes GPCRs with known ligands and focuses only on orphan GPCRs as potential candidates. Given that GPCRs can exhibit ligand promiscuity, any potential interaction with a characterized GPCR could have been overlooked. Lastly, our deorphanization strategy does not provide evidence for a physical interaction between ligand and candidate receptor. Future experiments must therefore involve biochemical analyses of the potential physical association between C-peptide and GPR146, including radioligand binding experiments, fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) assays, co-immunoprecipitation or pull down experiments, and re-evaluation of fluorescence co-localization using super resolution microscopy. The application of these tools has proven difficult for the study of the interaction of C-peptide with cell surface binding partners, because, in our hands, tagged forms of C-peptide (e.g. with fluorescent labels or biotin), which are necessary for most of the aforementioned techniques, do not exhibit biological activity in that they do not initiate signaling cascades normally observed in our cell systems. Furthermore, C-peptide appears to signal via an interaction with a receptor complex rather than with a signal receptor alone, and these protein–protein interactions appear to be transient or weak. In addition, we have found that some of the effects of C-peptide are dependent upon the glucose concentration of the culture medium and the relative concentration of insulin. These limitations could have important implications for understanding the role of C-peptide in normal physiology and in pathophysiological conditions, such as in diabetes-associated eye diseases.

C-peptide and the retinal pigment epithelium: effect of glucose and insulin on C-peptide signaling

Diabetic eye disease remains one of the major causes of visual impairment in the developed world. The etiology of diabetic eye disease is multifactorial and involves multiple pathological processes, including neovascularization and macular edema. Central to the development of diabetic macular edema is the retinal pigment epithelium (RPE), a monolayer of epithelial cells that forms the blood–retinal barrier and has essential metabolic roles in the retina [93]. In diabetes, the tight junctions between RPE cells are compromised, leading to blood–retinal barrier breakdown, leakage of excess fluid into the retina, and macular edema [94]. Although the mechanisms underlying RPE cell dysfunction have not been fully elucidated, over-secretion of vascular endothelial growth factor (VEGF) is

thought to be involved, as high levels of retinal VEGF have been associated with the disruption of RPE tight junctions [95].

C-peptide has been shown to reverse blood flow abnormalities and protect against VEGF-mediated damage in the retina in the setting of experimentally induced diabetes [96, 97]. These data suggest that C-peptide could be used as an effective agent for the treatment of diabetic eye disease. Indeed, plasma levels of C-peptide were inversely correlated with the incidence of diabetic retinopathy in patients with type 1 diabetes [98–100]. However, in order to specifically evaluate the utility of C-peptide in the treatment of diabetic macular edema, we evaluated its ability to influence RPE cell biology. We found that GPR146 is expressed in the RPE and that C-peptide increases cFos mRNA expression in the human RPE cell line ARPE-19 (Fig. 5), as measured by quantitative PCR (qPCR). Importantly, the effect of C-peptide on cFos mRNA expression appeared to be dependent upon extracellular glucose concentration, as incubation with high glucose (25 mM, equivalent to a blood glucose concentration of approximately 450 mg/dL) for 24 h prior to exposure of ARPE-19 cells to C-peptide inhibited C-peptide-induced cFos mRNA expression. C-peptide has been shown to activate 5' adenosine monophosphate-activated protein kinase (AMPK) in human umbilical vein endothelial cells (HUVECs), and C-peptide-induced inhibition of reactive oxygen species (ROS) was mediated by this activation [101]. We therefore propose that at least some of the effects of C-peptide are dependent upon extracellular glucose concentration due to the interaction between C-peptide-initiated signaling cascades and AMPK activation, as AMPK functions as a metabolic sensor by detecting and reacting to changes in the AMP:ATP ratio within cells [102]. Given that the release of C-peptide from beta cells is tightly regulated by blood glucose levels, we also predict that the dependency of its effects on glucose concentration is not limited to ARPE-19 cells and HUVECs, but rather is a phenomenon common to many if not most C-peptide-responsive cell types.

Because C-peptide is co-released with insulin in equimolar amounts, and C-peptide and insulin have been shown to initiate similar signaling cascades, including insulin receptor substrate 1 (IRS-1)- and Akt-mediated signaling events [103], it is possible that C-peptide and insulin interact with respect to their cellular activities and functions. Co-exposure of C-peptide and insulin in physiologically relevant ratios appeared to be critical for low oxygen tension-induced ATP release from human erythrocytes [87, 88]. Exposure of erythrocytes to either C-peptide or insulin alone was insufficient to restore ATP release and actually proved detrimental with regard to this function. We therefore tested the potential interaction between C-peptide and insulin in RPE cells. We found that co-treatment with C-peptide and insulin dramatically enhanced cFos mRNA expression in ARPE-19 cells compared to cells incubated with C-peptide alone (Fig. 5) and, importantly, this effect was reversed in the presence of a high glucose concentration (25 mM). Thus, C-peptide and insulin may interact in RPE cells, and this interaction may be important for the functions of both peptides.

However, these observations do not explain how C-peptide and insulin are interacting. Several possibilities exist. C-peptide and insulin could be interacting at the peptide level, perhaps acting as allosteric modulators of each other's receptors. Alternatively, C-peptide- and insulin-initiated signaling cascades could be interacting intracellularly at the levels of secondary signaling molecules (e.g. Akt). We propose that the putative C-peptide receptor,

GPR146, engages in G protein switching, depending in part on the concentration of insulin relative to C-peptide (hypothesis outlined in [84]). In this way, C-peptide, acting through its receptor, could either enhance or dampen insulin signaling through the activation or inhibition of appropriate downstream signaling molecules. This hypothesis could also explain discrepancies regarding the breadth of C-peptide-initiated signaling cascades, which range from the activation of protein kinase C (usually associated with $G\alpha_q$) to pertussis toxin-sensitive events (indicating involvement of $G\alpha_i/o$). Regardless, the potential interaction between C-peptide and insulin should be carefully considered when designing experiments to evaluate the biology of either peptide, because this interaction could have important implications for the function of these peptides in the RPE and other cell types as well.

C-peptide and RPE cell transdifferentiation

In addition to its metabolic roles in the retina, the RPE possesses the unique ability to transdifferentiate into other retinal cell types, including photoreceptors and ganglion cells [104–107]. The physiological significance of RPE transdifferentiation has not been fully elucidated, but this ability could be involved in retinal repair, with transdifferentiating RPE cells serving as a ‘stopgap’ when other retinal cell types have been damaged [108]. For example, injury to Bruch’s membrane or the endothelium of the choriocapillaris might in part drive the morphogenesis of RPE cells as a protective mechanism. In diabetes, aberrations in this process, including diminished activity of the Na^+/K^+ ATPase [109], lead to the transdifferentiation of RPE cells into fibroblasts and myofibroblasts that can form epiretinal membranes that contribute to retinal detachment [110]. The secretion of growth and angiogenic factors from these fibroblasts, including VEGF, could induce further damage to the retina, thus initiating a vicious cycle that spirals toward the progression of diabetic eye disease.

C-peptide has been shown to normalize Na^+/K^+ ATPase activity in multiple cell types [83, 111], therefore we hypothesized that exposure to C-peptide would prevent high glucose-induced transdifferentiation of ARPE-19 cells to a mesenchymal-like phenotype. To test this hypothesis, ARPE-19 cells were incubated with normal (5.5 mM) or high (25 mM) glucose for 30 days with or without 1 nM C-peptide, with weekly medium replenishment. Changes in expression of RPE-specific genes were evaluated using qPCR (Fig. 6). These experiments yielded two important findings concerning the role of C-peptide in RPE cell biology. First, C-peptide appeared to enhance expression of genes necessary for RPE cell function (Fig. 6), including *ZO-1* (zona occludens 1), which encodes a tight junction protein that is essential for the function of the RPE as part of the blood–retinal barrier [112], and *MITF* (microphthalmia-associated transcription factor), which is a transcription factor that regulates the expression of genes essential for RPE cell identity [113]. Thus, C-peptide may protect against diabetic macular edema at least in part by maintaining RPE cell identity and protecting against RPE cell transdifferentiation. Secondly, in agreement with our previous data showing that C-peptide-induced changes in cFos mRNA expression were dependent upon extracellular glucose concentration (Fig. 6), the effect of C-peptide on gene expression changes were also modulated by the concentration of glucose in the culture medium. For example, while C-peptide significantly enhanced *ZO-1* and *MITF* mRNA expression and

inhibited RLBP-1 mRNA expression under normal glucose (5.5 mM) conditions, this effect was lost in cells cultured in high glucose (25 mM). Notably, although C-peptide had no effect on VEGF receptor 2 (VEGFR2) mRNA expression in low glucose-exposed cells, cells cultured in high glucose exhibited a significant increase in VEGFR2 mRNA expression, which could have important implications for the functionality of VEGF on the RPE. Thus, the modulatory action of C-peptide on gene expression appears to be dependent upon extracellular glucose concentration, perhaps reflecting an interaction with AMPK-mediated signaling cascades. However, further experiments are required to evaluate the signaling mechanisms underlying this important action of C-peptide.

These data suggest that experiments to evaluate the cellular effects of C-peptide must be carefully planned to control for glucose concentration and relative insulin levels. Likewise, these findings could have important implications for the development of C-peptide-based therapeutics, particularly with respect to plasma insulin and glucose levels at the time of C-peptide administration. Regardless of these difficulties, C-peptide remains a promising target for the treatment of diabetes-associated complications, including diabetic retinopathy, nephropathy, and neuropathy. However, until the full identity of the C-peptide signalosome is elucidated, the role of C-peptide in normal physiology cannot be fully understood. Evaluation of the physiological relevance of C-peptide and its receptor or receptor complex would not only enhance our understanding of the role of endocrine function in the maintenance of the microvasculature, but would likely lead to the development of novel therapeutic options for both type 1 and type 2 diabetic patients suffering from microvascular dysfunction.

Conclusions

GPCRs are the target of numerous drugs, including agents currently being developed as adjuvant therapy in diabetes. Over 70 GPCRs have been identified that as yet have an unidentified ligand. These orphan GPCRs represent a significant untapped source of therapeutic potential for diabetes and its complications, but first their endogenous ligands must be identified. Current deorphanization strategies have had major successes, including the use of the deductive ligand–receptor matching strategy to identify GPR146 as a candidate receptor for proinsulin C-peptide. However, further work is necessary to elucidate the full signalosome of C-peptide, which should facilitate the development of future C-peptide-based therapeutics. Future deorphanization of additional orphan GPCRs could yield important novel options for the treatment of diabetes and its complications.

Acknowledgments

The authors would like to thank Dr. John Wahren for many useful discussions and comments during the preparation of this manuscript. GLCY is supported by a grant from the National Institutes of Health (# HL121456). GRK is supported by the Edward Doisy Research Fund and the President's Research Fund.

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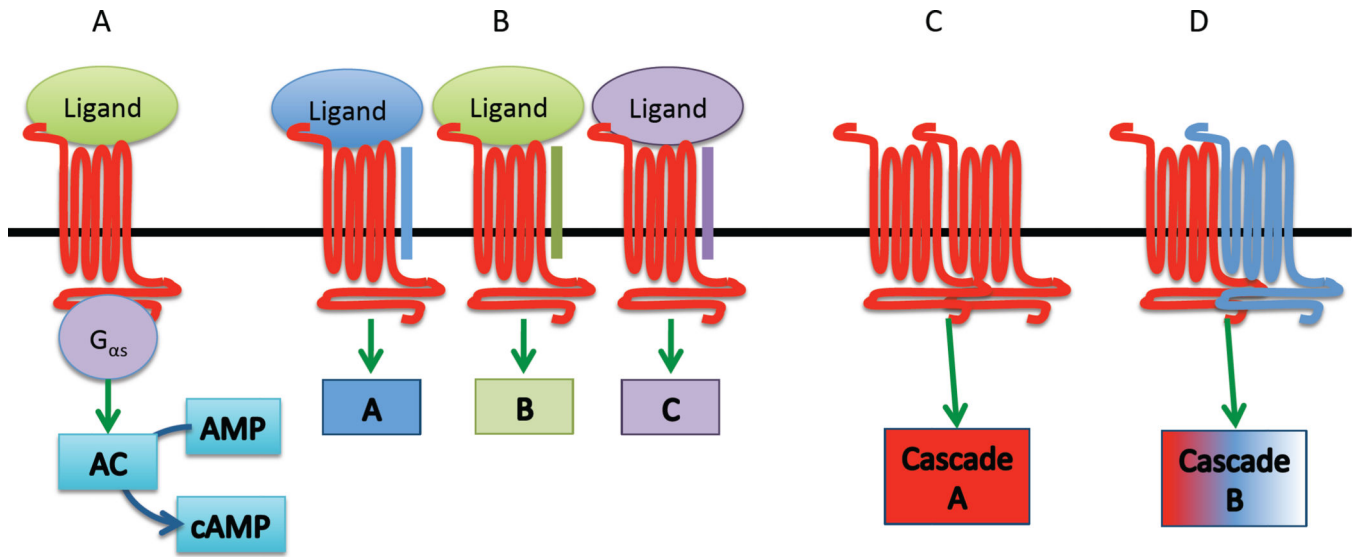
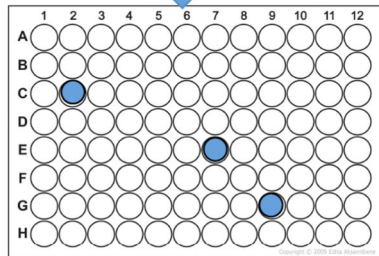


Fig. 1. The complexity of G protein-coupled receptor (GPCR) signaling. (A) Canonical GPCR signaling via a stimulatory G alpha protein. (B) Association with receptor activity-modifying proteins (RAMPs) (blue, green, and purple bars) changes the ligand specificity of some GPCRs. Downstream signaling cascades may be altered as well (represented by colored boxes). Some GPCRs form homodimers (C) or heterodimers with other GPCRs (D), which can change ligand and/or downstream signaling molecule specificity.

Forward Pharmacology

Functional Activity of
(e.g., Tissue Extracts)



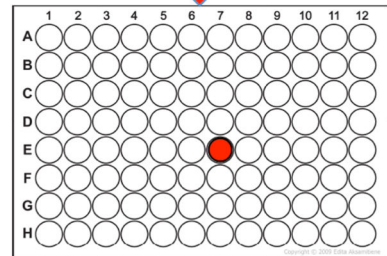
Identification of Lead Compounds
Or Potential Interacting Receptors



Target Identification

Reverse Pharmacology

Target Identification
(e.g., Bioinformatics)



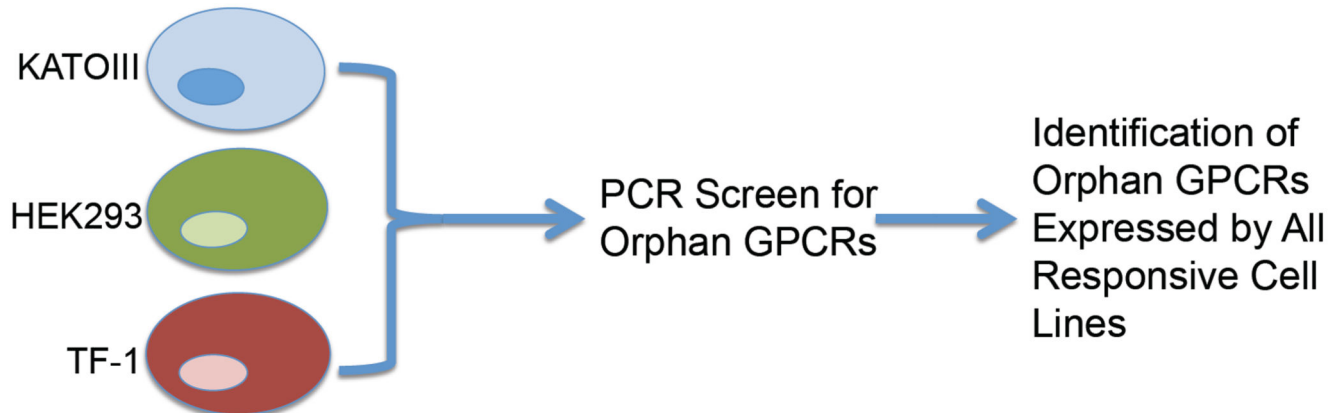
High-Throughput Screen To
Identify Potential Receptors



Functional Activity In Vitro
And In Vivo

Fig. 2.
Drug target discovery and receptor deorphanization strategies.

Identification of Candidate Receptors



Evaluation of Candidate Receptors

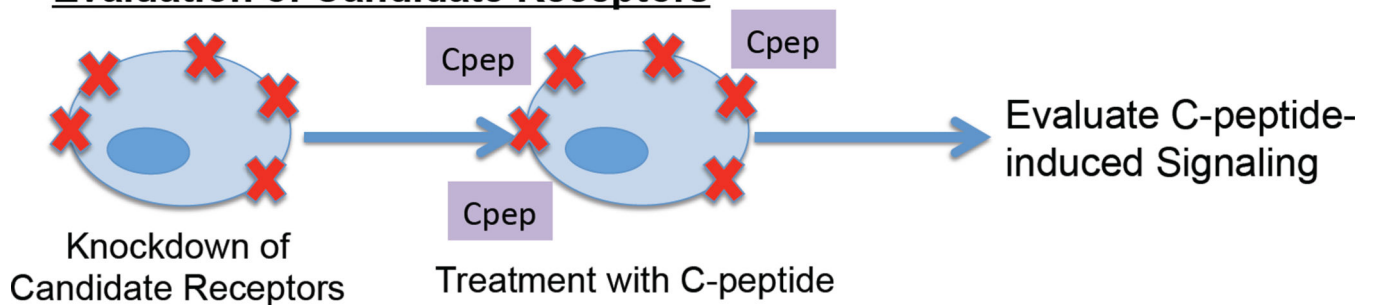


Fig. 3.

Utilization of the deductive ligand–receptor matching strategy for the identification of C-peptide receptor candidates. C-peptide-responsive cell lines were screened for the expression of orphan G protein-coupled receptors (GPCRs). Orphan GPCRs expressed by all responsive cell lines were then knocked down individually using siRNA in KATOIII cells. Cells were treated with C-peptide, and C-peptide signaling was assessed. Knockdown of GPR146 resulted in loss of C-peptide-induced signaling, and thus was considered the best candidate for the C-peptide receptor.

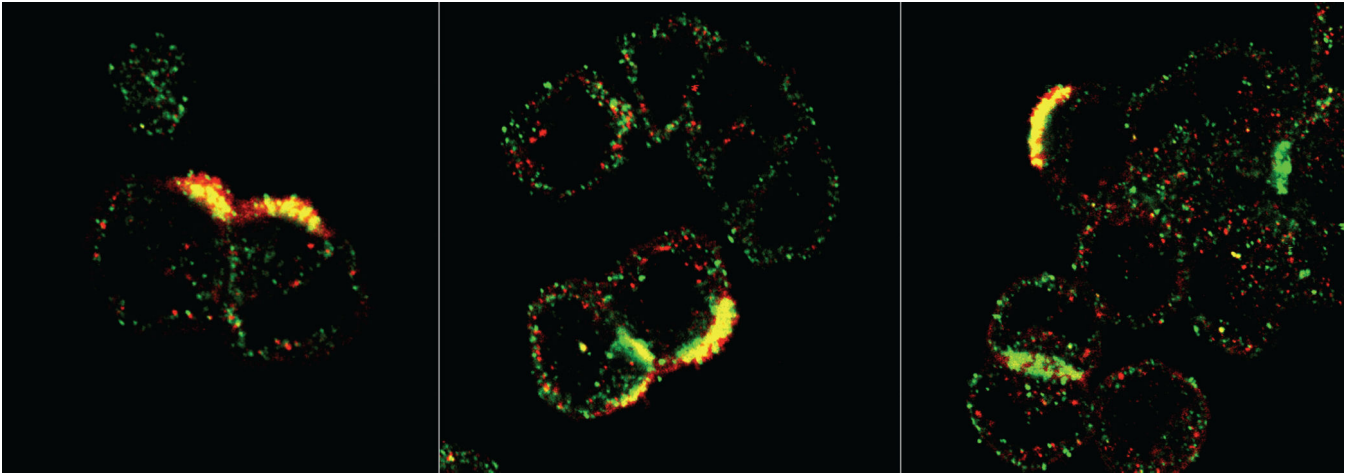


Fig. 4. Co-localization of C-peptide and GPR146 on KATOIII cell membranes. KATOIII cells were co-incubated with 1 nM C-peptide and 1 nM insulin and stained using antibodies against C-peptide (red) and GPR146 (green). Co-localized particles appear yellow. Representative images from three visual fields.

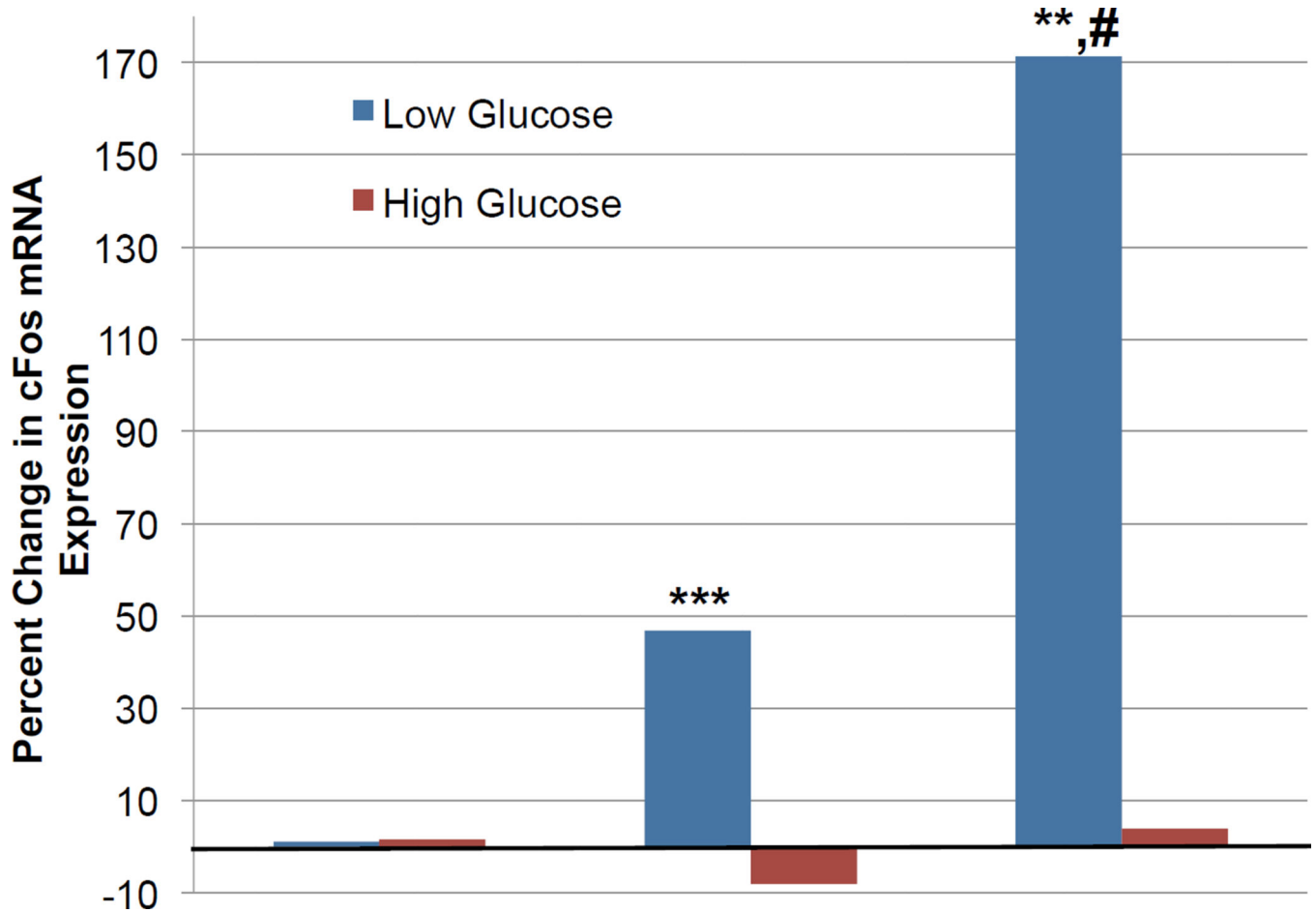


Fig. 5.

C-peptide-induced cFos mRNA expression in the human retinal pigment epithelial cell line ARPE-19. ARPE-19 cells were incubated in either low (5.5 mM) or high (25 mM) glucose for 24 h prior to exposure to vehicle, 1 nM C-peptide, or 1 nM C-peptide with 1 nM insulin for 1 h. Cells were lysed, RNA isolated, and changes in cFos mRNA expression evaluated using quantitative PCR. ** $P < 0.01$, *** $P < 0.001$ vs. low glucose, vehicle-treated cells; # $P < 0.01$ vs. low glucose, C-peptide-treated cells.

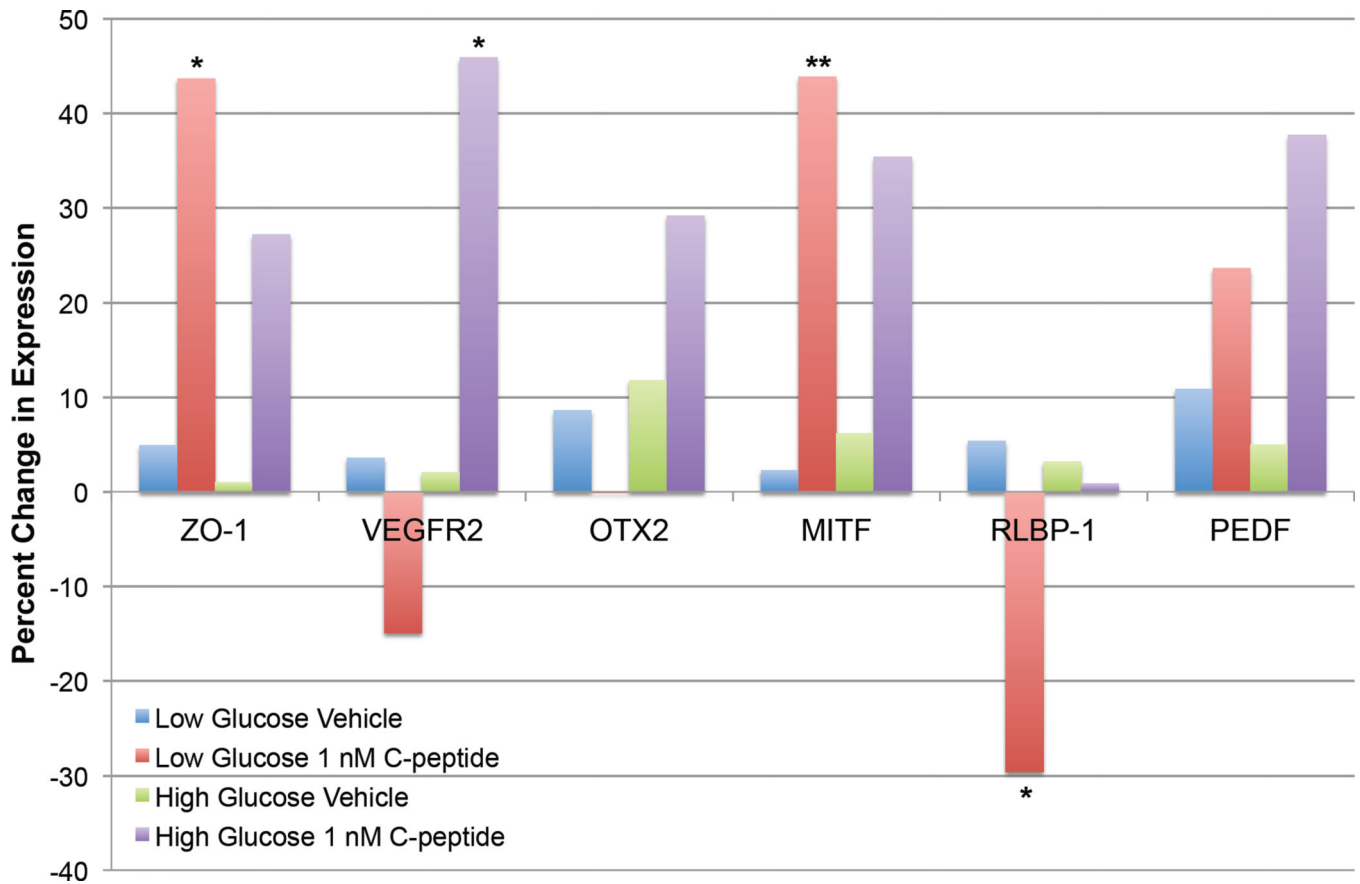


Fig. 6.

C-peptide and retinal pigment epithelium (RPE) cell gene expression changes in a transdifferentiation assay. ARPE-19 cells were incubated in either low (5.5 mM) or high (25 mM) glucose for 30 days with or without 1 nM C-peptide. Medium +/- C-peptide was replenished weekly. To evaluate C-peptide-induced changes in gene expression, cells were lysed, RNA isolated, and changes in gene expression evaluated by quantitative PCR using primers specific against genes produced by RPE cells, including the tight junction protein zona occludens 1 (*ZO-1*), the VEGF receptor 2 (*VEGFR2*), the transcription factor orthodenticle homeobox 2 (*OTX2*), the transcription factor microphthalmia-associated transcription factor (*MITF*), which is involved in regulating RPE cell identity, retinaldehyde-binding protein 1 (*RLBP-1*), and pigment epithelium-derived factor (*PEDF*). Under low glucose conditions, C-peptide significantly altered the mRNA expression of several genes, including *ZO-1*, *MITF*, and *RLBP-1*. However, this effect was lost in cells cultured in high glucose, suggesting that C-peptide-induced changes in gene expression are dependent upon glucose concentration, perhaps reflecting an interaction with AMPK-mediated signaling cascades. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated control.

Table 1

GPCR	Targeting compound	Trade name (if applicable)	Action	Approval status	Reference	
GLPIR	Exenatide	Byetta, Bydureon	Incretin mimetic	Approved	[114, 115]	
	Liraglutide	Victoza, Saxenda	Incretin mimetic	Approved	[116]	
	Albiglutide	Tanzeum	Incretin mimetic	Approved	[117]	
	Dulaglutide	Trulicity	Incretin mimetic	Approved	[118, 119]	
	Semaglutide		Incretin mimetic	Phase III	[120, 121]	
	Lixisenatide	Lyxumia	Incretin mimetic	Phase III completed	[12–124]	
	Taspoglutide		Incretin mimetic	Phase III terminated	[125]	
	Insulin degludec and liraglutide	iDeg-Lira	Combination	Phase III	[126]	
	Insulin glargine and lixisenatide	LixiLan	Combination	Phase III completed	[123]	
GPR40	Alogliptin	Nesina	DPP4 inhibitor	Approved	[127, 128]	
	Saxagliptin	Onglyza	DPP4 inhibitor	Approved	[129]	
	Sitagliptin	Januvia	DPP4 inhibitor	Approved	[130]	
	Vildagliptin	Galvus	DPP4 inhibitor	Phase III	[131–133]	
	Linagliptin	Tradjenta	DPP4 inhibitor	Approved	[134]	
	Gemigliptin		DPP4 inhibitor	Phase III	[135–137]	
	Fasiglifam (Tak-875)		GPR 40 agonist	Phase III Terminated	Reviewed in [138]	
	D2R	Bromocriptine	Cycloset	Reduces HbA1C		[63, 70]
		C-peptide	Ersatta	C-pepR agonist	Phase IIb	[139]

GPCR, G protein-coupled receptor.