Unravelling the molecular complexity of GPCR-mediated EGFR transactivation using functional genomics approaches

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Abstract

To influence physiology and pathophysiology, G protein-coupled receptors (GPCRs) have evolved to appropriate additional signalling modalities, such as the activation of adjacent membrane receptors. The epidermal growth factor receptors (EGFRs) mediate important growth and remodelling actions of GPCRs, although the precise network of gene products and molecular cascades linking GPCRs to EGFRs (termed EGFR transactivation) remain incomplete. In this review, we present the current view of GPCR-EGFR transactivation, identifying the established models of receptor cross-talk. We consider the limitations in our current knowledge and propose that recent advances in molecular and cell biology technology, including functional genomics approaches, will allow a renewed concentration of our efforts on understanding the mechanism underlying EGFR transactivation. Using an

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unbiased approach to the identification of the molecules required for GPCR-mediated EGFR transactivation will provide a contemporary and more complete representation from which to extrapolate therapeutic control in diseases from cardiovascular remodelling to cancer.

**Introduction**

The G protein-coupled receptor (GPCR) family is one of the largest and most studied families of cell surface proteins, with almost 800 members identified in the human genome [1]. GPCRs form an important nexus between the extracellular and intracellular environment. They are essential regulators of cellular physiology and pathophysiology, and mutations or modifications of this receptor family are associated with aberrant cellular signalling and disease. GPCRs have proven excellent therapeutic targets for drug discovery, where it is estimated that some 40-50% of therapeutics used to treat disease specifically target their actions. Moreover, a large subset of the GPCR family remain as orphan receptors with no known agonists, that may also provide a potential source of novel therapeutic targets for the treatment of disease [2].

GPCRs are seven transmembrane spanning receptor proteins that are activated by a range of different stimuli that include proteins, peptides, lipids, amino acids, ions, biogenic amines, light and odorants (Figure 1). Upon agonist binding/activation, GPCRs undergo a conformational switch, whereby the receptor acts as a guanine nucleotide exchange factor (GEF) catalysing the exchange of guanosine diphosphate (GDP) on the α-subunit of the heterotrimeric G protein complex to guanosine triphosphate (GTP) (Figure 1). Exchange of GDP to GTP dissociates the heterotrimeric G protein complex into two components: the Gα-subunit coupled to GTP, and the Gβγ subunits. Adding to the complexity of G protein-dependent signalling are the four major members of the α-subunit family: Gαi, Gαs, Gα12/13.

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and Go4 that are responsible for eliciting different signalling responses. The Gβγ heterodimer is also capable of acting on ion channels and activating phosphatidylinositol 3-kinase (PI3K) and phospholipase C beta (PLCβ) to elicit various cellular effects [3-5]. The plethora of downstream signalling cascades that are activated in a ligand-dependent and ligand-independent manner can lead to changes in cellular growth and/or proliferation, migration and invasion, angiogenesis and survival (Figure 1) [6-11].

The activities of most agonist-activated GPCRs are regulated by GPCR kinases (GRKs). There are seven known mammalian GRKs (GRK1-7, reviewed in more detail in [12]) that phosphorylate the C-terminal tail of activated GPCRs, preventing further interaction with heterotrimeric G proteins and leading to termination of receptor signalling and receptor desensitisation. GPCR phosphorylation also facilitates recruitment of arrestin proteins (β-arrestin) that act as molecular chaperones, binding to the desensitised receptors and allowing endocytosis via clathrin-coated pits. During desensitisation and endocytosis, arrestins also act as scaffolding proteins to recruit a series of signalling and regulatory protein that add special and temporal complexity to GPCR function. A comprehensive overview of the role of arrestins in GPCR signalling can be found in [13].

While the general canonical, mitogenic signalling pathways activated by GPCRs are reasonably well defined [14], another less well understood, but equally important facet of GPCR signalling is their ability to appropriate the function of receptor tyrosine kinases. This receptor “hijacking” leads to the activation of important mitogenic signalling pathways downstream of these receptors. The first evidence implicating the activation of GPCRs in cross-talk with receptor tyrosine kinases was provided by Alex Ullrich and colleagues, demonstrating that stimulation of Rat 1 cells with various GPCR agonists (lysoosphatidic

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acid, LPA; endothelin-1 and thrombin) promoted the rapid phosphorylation of the epidermal growth factor receptor (EGFR) and the neu oncoprotein (ErbB2). This activation was inhibited when cells were treated with tyrphostin AG1478 (EGFR kinase inhibitor) or when a dominant negative EGFR was expressed in the cells [15]. Since then, GPCR cross-talk has been confirmed for a number of receptor tyrosine kinases, including platelet-derived growth factor receptor (PDGFR) [16, 17], vascular endothelial growth factor receptors (VEGFR) [18, 19], insulin-like growth factor receptor [20, 21] and fibroblast growth factor receptors (FGFR) [22]. In this review, we focus on the EGFR and ErbB family of receptor tyrosine kinases, recognising the central position they command in cell growth and remodelling in health and disease.

The ErbB receptor family

The ErbB receptors are a well-studied family of proteins which are comprised of four members: EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). Apart from ErbB2, the ErbB receptor family can be activated by a number of different ligands. Ligands specific to the EGFR include epidermal growth factor (EGF), amphiregulin and transforming growth factor alpha (TGF-α), while heparin-binding EGF (HB-EGF), betacellulin and epiregulin have affinities for both the EGFR and ErbB4. Neuregulins 1 and 2 can bind to ErbB3 and ErbB4, while neuregulins 3 and 4 have specificity for ErbB4 only (reviewed in more detail in [23, 24]). Upon ligand binding, ErbB receptors can either homo- or heterodimerise with other members of the ErbB family, leading to activation (phosphorylation) of the intrinsic tyrosine kinase domain located within the cell. The phosphorylation of the C-terminal tail of the receptors generates multiple docking sites, where different signalling adapter molecules such as growth factor receptor bound protein 2 (GRB2), Src homology 2 domain containing protein (SHC) and phospholipase Cγ (PLCγ)

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can bind and promote signalling scaffolds that lead to the activation of growth promoting signalling cascades, including the mitogen activated protein kinase (MAPK), PI3K-AKT and others important for cellular functioning.

Under normal physiological conditions, ErbB family members play an important role in embryonic development. For example, depending on the genetic background, mice lacking EGFR expression do not survive for more than 20 days post-natally and have defects in the development of skin, lung and gastrointestinal tract [25, 26]. Moreover, ErbB2, ErbB3 and ErbB4 are a requirement for neural and cardiac development, where embryonic lethality results in mice that are lacking expression of any of these receptors [27-31]. ErbB family members are also involved in the maintenance of normal physiological processes in the adult; it has been demonstrated that ErbB2 signalling is critical for the prevention of dilated cardiomyopathy [32, 33], and evidence suggests that ErbB4 is expressed in the adult rat heart [34], though its exact physiological function is unknown. Moreover, the focus of ErbB family members in adults concerns their strong implication in cancer, where aberrant expression and/or activity of these receptors have been associated with the development and progression of a variety of malignancies, including those of the breast, ovary, lung and gastrointestinal tract (reviewed by [24]).

Mechanistic insights into how GPCRs hijack ErbB receptor signalling

An important question to ask is how do GPCRs transactivate receptor tyrosine kinase signalling to drive growth and other cellular processes distal of the EGFR? From available evidence, the process is highly complex, and seems to be dependent on a number of factors, including the cell type, the type and class of the GPCR and the cellular environment. Transactivation of the EGFR by GPCR signalling has been suggested to occur through at

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least two different mechanisms, which are demonstrated in Figure 2. The first mechanism is the 'triple membrane-pass signalling' (TMPS) paradigm, where GPCR-mediated EGFR transactivation is dependent on the activation of membrane-bound matrix metalloproteases (MMPs) such as the ADAM (α disintegrin and metalloprotease) family members. These molecules are able to cleave EGF ligands and promote ligand shedding into the extracellular space. The EGF ligands (such as EGF, HB-EGF, neuregulins) are then able to bind to the EGFR and other EGFR family members and promote dimerisation and activation of the EGFR mediating the activation of signalling cascades including the mitogen activated protein kinase (MAPK) pathway and the PI3K-AKT pathway and elicit downstream cellular responses. ADAM and MMP-dependent EGFR transactivation has been observed to occur in various cell types after stimulation with agonists such as 5-hydroxytryptamine (5-HT) [35], angiotensin II (AngII) [36-41], bombesin [34], bradykinin [42], carbachol [34, 43, 44], endothelin 1 (ET-1) [36, 42, 44], gonadotropin-releasing hormone (GnRH) [45], LPA [34, 42-44], phenylephrine (PE) [36, 38, 46], thrombin [42], leptin [47], deoxycholic acid (DCA) [48], prostaglandin E2 [49], and 1-(1-Methylethyl) 1H benzotriazole-5-carboxylic acid (IBC293) [50]. Furthermore, the dependence on MMPs and ADAMs, in particular ADAMs 10, 12 and 17 (tumour necrosis factor-α-converting enzyme, TACE) in GPCR-mediated EGFR transactivation have been demonstrated to be important for the growth, development and progression of cancers, and also as a mechanism contributing to the pathogenesis of cardiovascular and kidney disease [40, 42-44, 51-61]. Given their broad and central role in many homeostatic mechanisms, some caution does need to be applied when interpreting data indicating a conclusive, specific role for these proteases in EGFR transactivation.

The second mechanism for transactivation of the EGFR by GPCR signalling involves direct phosphorylation of the EGFR post stimulation of the GPCR, that is intracellular in nature and can either be independent of or partially dependent on the activities of MMPs and EGF.
ligands. Considerable evidence suggests that stimulation of GPCRs can lead to the activation of second messenger molecules - which can include $\text{Ca}^{2+}$, PKC, Pyk2, Src, arrestins and reactive oxygen species (ROS) which in turn lead to the tyrosine phosphorylation and subsequent activation of the EGFR [55, 62-72]. Whether some or all of these alternative pathways in fact are coincident with, or involve, MMP/ADAM activation remains unclear.

Relevance of investigating GPCR cross-talk with EGFR signalling in disease

GPCR mediated EGFR transactivation is likely to be an important yet under-appreciated contributor to the molecular complexity of specific diseases associated with GPCR or EGFR dysregulation, such as cancer and cardiovascular disease. At the GPCR level, an example of this is the well-characterised angiotensin type 1 receptor (AT$_1$R), physiologically important for fluid and salt balance, yet is also implicated in pathological cardiac hypertrophy and hypertension, and more recently in cancer [73]. While many studies use in vitro based studies to investigate the role of AT$_1$R-EGFR transactivation [37, 40, 41, 66, 72, 74-76], Asakura and colleagues provided compelling in vivo evidence that cardiac hypertrophy was mediated by GPCR-EGFR transactivation (with the AT$_1$R being one of the GPCRs implicated), the phenotype of which could be blocked with an inhibitor of ADAM12 [36]. Furthermore, when an EGFR kinase inhibitor was using in rat aorta, there was attenuation in the level of AngII mediated protein synthesis (a measure of cellular growth), also suggestive of a role for AT$_1$R-EGFR transactivation in vivo [77]. While clinically available inhibitors exist for the antagonism of the AT$_1$R or reduction in angiotensin production to treat cardiac hypertrophy and hypertension (reviewed in [73]), these drugs produce a broad, non-selective antagonism that inhibits all aspects of AT$_1$R function. We predict that a deeper understanding of AT$_1$R (GPCR)-EGFR transactivation may provide a platform for developing pathway selective
inhibitors, thereby permitting retention of normal physiological signalling while modulating say unwarranted growth.

Similarly, the use of therapeutics targeting the activities of EGFR and ErbB family members are commonplace for cancers in which EGFR is dysregulated, for example, EGFR gene amplification. While their use in the treatment of various malignancies, such as lung, breast and ovarian cancers are well described, resistance to these treatments remains a large problem [24, 78, 79]. With the available evidence, GPCR cross-talk with EGFR signalling provides another avenue by which cancer cells could increase their survival, growth and metastatic potential. Specific examples of this include the activation of protease-activated receptor 1 (PAR1) by thrombin, which, in invasive breast cancer, leads to the transactivation of the EGFR and hyperactivation of ErbB family members, increased ERK1/2 phosphorylation and invasive potential of cells [54]. Similarly, in squamous cell carcinoma, stimulation of cells with LPA or carbachol results in increased shedding of amphieregulin; silencing of amphieregulin prevented GPCR-induced EGFR tyrosine phosphorylation, subsequently preventing cellular proliferation and migration [43]. Furthermore, squamous cell carcinoma cells derived from head and neck cancers (HNSCC) stimulated with GPCR agonists including LPA lead to the activation of the EGFR and MAPK pathways, increasing cellular proliferation and motility [80]. This is another instance where specific targeting of the molecules underpinning GPCR-mediated EGFR transactivation may circumvent the activation of the EGFR and EGFR family members to prevent increased growth signalling in cancer.

Moreover, patients which have mutations or SNP variations in receptors that lead to changes in expression, post-translational modification and/or activity and are thus refractory to standard antagonist therapy may also benefit clinically from drugs designed to target the intermediate molecules involved in GPCR mediated EGFR transactivation. For example, a

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recent study by Oganesian and colleagues demonstrated that ectopic expression of the naturally occurring G247R SNP (predisposing individuals to hypertension) in the α(1a)-adrenergic receptor in Rat-1 cells leads to an agonist-independent increase in cellular proliferation, which is dependent on β-arrestin 1 and MMP/ADAM-dependent transactivation of the EGFR [81]. While further investigation is required, this observation may possibly explain why certain individuals are predisposed to diseases such as hypertension, and may go some way to understanding why some patients are refractory to common therapeutics, thus emphasising the need to develop alternative therapeutic strategies to treat some forms of disease that are dependent on GPCR-mediated EGFR transactivation.

Is functional genomics the key to unlocking a more complete mechanism underlying GPCR mediated EGFR transactivation?

Since the discovery of the GPCR-EGFR transactivation phenomenon in 1996, hypothesis-driven candidate approaches have identified a handful of molecular candidates involved in this process. Yet, the progress into further understanding the key molecules that are critical to linking GPCR activation with the transactivation of the EGFR has started to wane in recent years. Why is this? With the complexity of different GPCRs and cellular model systems, a hypothesis-driven approach may be laborious and costly, and lead to failure to identify relevant molecular targets. Presumably, in many cell systems, transactivation of the EGFR is a variation on a theme – involving one or more of a number of ADAMs/MMPs to shed one or more of the 13 or so EGF ligands to activate one or more of the ErbB receptors, either directly or via dimerisation. The question is whether this combinatorial potential is sufficient to explain GPCR-EGFR transactivation in all situations? Are there generic upstream molecule(s), such as a signalling-regulated kinase, that are key components linking the activation of a GPCR to the activation of the EGFR? Or are there multiple positive and
negative influences on EGFR transactivation that are as yet unidentified? A comprehensive and contemporary model is required, however, the standard candidate driven approaches are limiting in this regard. One possible solution is to take a functional genomics-based approach to unbiasedly identify the molecular candidates to paint a more complete picture of the molecular process that is GPCR mediated EGFR transactivation.

In 2001, when the human genome sequence was published [82, 83], it provided a vast amount of genetic information. However, a large proportion of the gene sequences identified had no ascribed function. In the post-genomics era, new high-throughput tools were developed to assess the function of genes in specific biological systems, now known as the field of ‘functional genomics’. While many different technologies have been developed to assess gene function, a key finding in the functional genomics revolution was the development of RNA interference (RNAi) technologies, first demonstrated in C. elegans as a normal mode of regulating gene expression [84]. With advancements in technology, this process could also be manipulated to ‘knock down’ specific genes of interest in order to further determine the function of a gene. Since then, technologies have evolved to perform genome-wide RNAi screens that are capable of interrogating the role of each individual gene in the genome within a specific biological context. While the types of RNAi screens that can be performed is beyond the scope of this review (more comprehensive information about the types of assays that can be performed and assay design can be found in [85-87]), we discuss the use of chemically synthesised, double-stranded short interfering RNA (siRNA) based high-throughput screening approaches.

Functional screens using chemically synthesised siRNAs are usually performed in 96 or 384 well formats. In most cases, screens are designed so that the knockdown of a single candidate of interest is performed in an individual well of the microplate, which is one of the greatest advantages of using this particular methodology. Genome-wide siRNA libraries for human
and other model organisms (such as mouse) are commercially available, and these libraries can assess the function of anywhere between 18,000 to 22,000 protein coding genes within a defined biological context [87]. But screening is far from straightforward – it requires extensive assay development; a robust and quantifiable assay output or endpoint; sophisticated robotic platforms to perform complex liquid handling steps; a high level of bioinformatic support to be able to identify the strongest candidates from the screen; and finally, extensive validation of these candidates. These are all critical factors in order to be able to assess the biological functions/pathways in which these candidates function. The first published genome-wide functional siRNA screen was performed in 2007 to identify candidates that reduced the viability of non-small cell lung carcinoma cells in the presence of paclitaxel (synthetic lethal screen) [88]. Many others have followed, addressing important biological questions, for example, the discovery of host factors in influenza virus replication [89], the identification of genes that are involved in human epithelial cell migration [90] and in cell division [91].

As with all high-throughput functional screening formats, there is a risk of false negatives, i.e., not all the contributing gene products are identified. Specifically in the case of GPCR-mediated EGFR transactivation, the high redundancy of GPCRs, ligands, ADAMs, MMPs and ErbB family members may increase the difficulty of identifying novel candidates within the system. In spite of these confounding issues, the long-term goal should be to identify novel candidates that have had no previous association with AT1R-EGFR or more generally, GPCR-EGFR transactivation, using an unbiased functional screening approach, which may serve as therapeutic targets for treatment in diseases including cardiovascular disease and cancer. With this in mind, we have proceeded to generate a stable human cellular model of AT1R EGFR transactivation using mammary epithelial cells, where stimulation of cells with
AngII leads to the robust activation of EGFR and ERK1/2 and can be blocked with pharmacological inhibitors of EGFR activity. We have used this cellular model to perform siRNA screening using a high-throughput robotics platform, with microplate-based Alphascreen SureFire ERK1/2 activation assays (the technique of which is described in [92]). This approach for quantifying ERK1/2 activation in cells after GPCR stimulation has been previously used for GPCRs including GPR40, GPR41, GPR43, GPR55 and the calcium-sensing receptor (CaR) [93-95]. In our hands, we can successfully quantify robust and significant changes in ERK1/2 activation after AngII stimulation of our cellular system and screens are currently ongoing.

Summary

In addition to canonical signalling pathways elicited by GPCRs, in certain contexts, GPCRs are also capable of engaging in receptor tyrosine kinase (EGFR) ‘cross talk’ to drive various cellular physiological and pathological outcomes. While a number of molecules have been discovered to play a role in the mechanism underlying this process, in more recent times, progress in this field has stalled, and much detail regarding the complete mechanism is still lacking. In order to improve our understanding of this field, we propose a shift from the candidate-based approaches to using high-throughput unbiased functional genomics platforms to identify novel molecular candidates that are involved in GPCR-EGFR transactivation. These molecular candidates may serve as alternative therapeutic strategies to specifically treat diseases where this mechanism is relevant and therefore enhance patient outcomes.

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Inactive

**Agonists including:**
- Peptides, proteins and lipids
- Amino acids and nucleotides
- Bioamines and ions
- Light and photons
- Odorants

**Cell membrane**

**Pl3K, Adenylyl cyclase**

**J.cAMP**

**RhoGEF**

**DAG, PKC**

**Ins(1,4,5)P_3, ↑Ca^{2+}**

**Cellular responses including:**
- Growth and/or proliferation
- Differentiation
- Migration/invasion
- Angiogenesis
- Survival

Active

**GPCR**
Figure 1: Activation of G protein-coupled receptors (GPCRs) leads to diverse signalling outcomes. GPCRs are activated by a diverse range of agonists that include peptide hormones, proteins, lipids, amino acids, nucleotides, bioamines, ions, light and odorants. Upon agonist binding and/or activation, the receptor undergoes a conformational change that allows the exchange of guanidine diphosphate (GDP) to guanidine triphosphate (GTP) on the Go subunit of the heterotrimeric G protein complex (also containing Gβ and Gγ subunits). The binding of GTP leads to the dissociation of the heterotrimeric G protein complex into the Go subunit and the Gβγ dimer. The GTP-bound Go subunit can then interact with downstream signalling effectors leading to the activation of various second messenger molecules (depending on the class of Go subunit). Subsequently, other signalling mechanisms are activated and are responsible for eliciting cellular responses that include cellular growth and/or proliferation, differentiation, migration and invasion, angiogenesis and survival signalling. The Gβγ dimer is also capable of activating PKB and PLCβ and also acting upon ion channels. Diagram adapted from [96-98]. Ca2+, calcium ion; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; GRK, G protein receptor kinase; Ins(1,4,5)P3, inositol 1,4,5 trisphosphate; P, inorganic phosphate; PT3K, phosphatidylinositol 3-kinase; PLCβ, phospholipase C beta; PKA, protein kinase A; PKC, protein kinase C; RGS, regulator of G protein signalling; RhoGEF, Rho guanine nucleotide exchange factor.

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Figure 2: GPCR-mediated transactivation of the EGFR. In specific cell types and under certain conditions, GPCR stimulation can lead to the activation of second messenger molecules, including PKC, Src, Pyk2, protein tyrosine kinases (PTKs), calcium (Ca^{2+}) and other unknown molecules. Transactivation of the EGFR by GPCR signalling can occur through two different mechanisms; the ‘triple membrane pass signalling’ (TMPS) paradigm, where second messengers can activate membrane-bound matrix metalloproteases (MMPs) such as the ADAM (a disintegrin and metalloprotease) family members which cleave EGF ligands that are able to bind to the EGFR and other EGFR family members and promote dimerisation and activation of the EGFR, subsequent activation of signalling cascades including the mitogen activated protein kinase (MAPK) pathway and the PI3K-AKT pathway and ultimately leads to cellular responses including growth, migration, invasion and survival. Diagram adapted from [73].