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Review

Dissection of Aberrant GPCR Signaling in Tumorigenesis – A Systems Biology Approach

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Abstract. *The superfamily of G-protein-coupled receptors (GPCRs) is one of the largest mammalian protein families. It is involved in signal transduction and participates in the regulation of normal physiological function and pathological progression of a range of diseases. Recent studies have demonstrated that many aberrant GPCRs and their ligands are associated with tumorigenesis, angiogenesis and metastasis, which provides promising opportunities for drug discovery for cancer prevention and treatment. It is necessary to search for drug targets such as ligands of unknown GPCRs and better modulators of known GPCRs using high throughput screening approaches. Here, we review recent research advances in the identification of novel GPCRs and their protein interactions.*

G-Protein-coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, 7TM receptors, heptahelical receptors, serpentine receptor, and G- protein-linked receptors (GPLR), constitute the largest family of cell-surface molecules with over 500 members identified in the human genome (1). GPCRs can sense a wide variety of extracellular stimuli (such as ions, biogenic amines, purines, lipids, peptides and proteins) and transduce these signal into the cells by activating a cascade which is initiated by catalyzing GDP-GTP exchange on heterotrimeric G proteins, participating in the regulation of major biological processes such as secretion, neurotransmission, growth, cellular differentiation and the immune response (2-4). In mammalian biology, GPCRs can be classified into three major receptor families based on sequence similarity, as shown in Table II. The class A rhodopsin family is the largest family of GPCRs and accounts for over 80% of all GPCRs (4). This family contains the rhodopsin, adenosine, melanocortin, neuropeptide, olfactory, chemokine, and melatonin receptors, amongst others (5, 6), and is characterized by several conserved residues in their transmembrane helices and a palmitoylated cysteine in the *C*-terminal tail. The class B secretin receptor family is a relatively small group that comprises about 60 members, including the gastrointestinal peptide hormone family (secretin, glucagon, vasoactive intestinal peptide, growth hormone-releasing hormone, calcitonin, and parathyroid hormone) and corticotrophin-releasing hormone receptors (4-6). These receptors are characterized by the presence of a large *N*-terminal domain containing several well-conserved cysteine residues. The class C metabotropic glutamate receptor family comprises around two dozen GPCRs, such as metabotropic glutamate receptors (mGluR), the calcium-sensing receptor (CaSR), GABAB receptors and also some potential taste receptors, and is characterized by a very long *N*-terminal domain that appears to be sufficient for ligand binding (4, 7). Additionally, several other families have been proposed. For example, on the basis of phylogeny, the human GPCRs have been divided into five families (rhodopsin-like, secretin, adhesion, glutamate, and frizzled/taste2) (8). Alternatively, using sequence homology and functional similarity, these receptors can be divided into six classes (class A-F) including rhodopsin-like, secretin-like, metabotropic, glutamate, pheromone, cAMP, and frizzled/smoothened family (9-12). The frizzled/smoothened receptor group appears to be particularly clinically relevant. Frizzled and smoothened receptors are key regulators of animal development that signal through the Wnt and Hedgehog signaling pathways, respectively (9).

GPCRs and their respective accessory proteins, as well as their signaling pathways, represent the largest group of

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Table I. *Abbreviations*.

2D-DIGE: Two-dimensional difference gel electrophoresis;
2-DE: Two-dimensional gel electrophoresis;
5-HT2C: 5-hydroxytryptamine 2C;
AC(s): Adenylyl cyclase(s);
APA: Aldosterone-producing adenomas;
BCC: Basal cell carcinoma;
BMSC: Bone marrow stromal cell;
cAMP: Adenosine 3', 5'-monophosphate;
CaSR: Calcium-sensing receptor;
CGH Arrays: comparative genomic hybridization arrays;
CTGF: Connective tissue growth factor;
DAG: Diacylglycerol;
EDG: Endothelial differentiation gene;
EL3: Third extracellular loop 3;
EPAC: Exchange factor activated by cAMP;
EST: Expressed sequence tag;
FACS: Fluorescence activated cell sorting;
FRET: Fluorescence resonance energy transfer;
GDP: Guanosine diphosphate;
GFP: Green fluorescent protein;
GIT: G-Protein-coupled receptor kinase-interacting protein;
GPCRs: G-Protein-coupled receptors;
GRKs: G-Protein-coupled receptor kinases;
GRO α-γ: Growth regulated oncogene α-γ;
GSK-3β: Glycogen synthase kinase-3β;
GTP: Guanosine triphosphate;
GTPase: Small guanosine triphosphatase;
Gαt: Transducin;
HCC: Hepatocellular carcinoma;
hH1R: Human histamine H1 receptor;
HTS: High-throughput screening;
ICAT: Isotope-coded affinity tagging;
IKAch: Ion channel activation;
IP3: Inositol triphosphate;
iTRAQ: Isobaric tags for relative and absolute quantitation;
LPA: Lysophosphatidic acid;
MAPK: Mitogen-activated protein kinase;
MCP-1: Monocyte chemoattractant protein 1;
mGluRs: Metabotropic glutamate receptors;
MRM: Multiple-reaction monitoring;
mRNADD-PCR: mRNA differential display polymerase chain reaction;
MS: Mass spectrometry;
MudPIT: Multidimensional protein identification technology;
PCAs: Protein complementation assays;
PIP2: Membrane D2-phosphatidyl inositol;
PKA: Protein kinase A;
PKC: Protein kinase C;
PI3Ks: Phosphoinositide 3-kinases;
PLC: Phospholipase C;
PLCβ: Phospholipase Cβ;
PTX: Pertussis toxin;
RHOA: Ras homolog gene family, member A;
ROCK: Rho-associated protein kinase;
SAGE: Serial analysis of gene expression;
SCC: Squamous cell carcinoma;
SDF-1: Stromal cell-derived factor;
SILAC: Stable isotope labeling by amino acids in cell culture;
TM7: The seventh transmembrane helix

molecules currently targeted by pharmaceutical drugs (13). Currently, GPCRs are associated with almost every major therapeutic category or disease class, including pain, asthma, inflammation, obesity, cancer, as well as cardiovascular, metabolic, gastrointestinal and central nevrous system diseases (14). Over 50% of the current therapeutic agents on the market are targeted towards GPCRs (15), including more than a quarter of the 100 top-selling drugs with annual turnover in the range of several billion US dollars (16). However, data from the Human Genome Project indicates that there are approximately 1000 genes encoding GPCRs; to date only about 200 of these possess annotated ligands and functions (17). Searching for ligands of the orphan GPCRs and better modulators of known receptors will provide new opportunities in future drug discovery (18). In addition, due to the emergence of new technologies such as green fluorescent protein (GFP), fluorescence resonance energy transfer (FRET), protein complementation assays (PCAs), and GPCR microarrays, ligands and modulators of GPCRs can be screened in a high throughput manner allowing a better understanding of the significance of GPCRs as drug targets.

The number of documented interactions involving GPCRs is rapidly growing, making the analysis of the functional significance of GPCR complexes and the kinetics of these interactions the next major challenge (19). The intricate biology of GPCR complexes leads to context-adapted biological outcomes which rely on emerging system-level properties that cannot be predicted from the individual components of the induced networks (20). However, systems biology has emerged in the last decade as a powerful new paradigm for research in the life sciences, which holds promise as a systematic approach to interpreting and understanding the complexity and dynamics of GPCR signaling complexes. This review highlights some currently available system biology methodologies based on omics approaches, including genomics, transcriptomics and proteomics to identify novel GPCRs and their interactive proteins and study GPCR functions in various disease states, which will help provide a global view of the GPCR signal transduction network.

GPCR Signaling Pathways

All GPCRs have a common central core domain consisting of seven transmembrane helices connected by three intracellular loops and three extracellular loops. The length of the individual *N*-terminal and *C*-terminal domains are variable in different GPCRs (21). The basic signaling unit of a GPCR system comprises three parts: the receptor, the trimeric αβγ G protein, and an effector (22). Upon activation by extracellular ligands, GPCRs bind heterotrimeric GTP-binding proteins, which promote not only the release of GDP from the G protein α subunit and the exchange for GTP, but

Table II. *G-Protein-coupled receptor classification*.

Class	Subfamily
Rhodopsin family	Thrombin, GPR37/endothelin B-like, adenosine, platelet-activating factor, histamine, serotonin, interleukin-8, alpha adrenoceptors, beta adrenoceptors, neuropeptide Y, neurotensin, olfactory, C-X-C chemokine, endothelin, melanocortin, chemokine receptor-like, rhodopsin vertebrate, melatonin, lysosphingolipid and lysophosphatidic acid
Secretin receptor family	Secretin, glucagon, vasoactive intestinal peptide, calcitonin, parathyroid hormone, growth hormone-releasing hormone, corticotrophin-releasing hormone receptors
Metabotropic glutamate receptor family	GABAB receptors, the calcium-sensing receptor (CaSR), metabotropic glutamate receptorsI, II, and III, some potential taste receptors
Other	cAMP receptor family, frizzled/smoothened family, pheromone receptors family, other

*GPCR classifications according to sequence similarity are not all listed in this table. The following sources were used: KEGG (http://www.genome.jp/kegg/kegg2.html) and BIAS-PROFS (http://www.cs.kent.ac.uk/projects/biasprofs/index.html).

also dissociation of the GTP-bound α-subunit and βγ-dimer from the GPCR (23, 24). On one hand, dissociated Gα subunits can couple with an effector, such as adenylyl cyclase and phospholipase C β , or an ion channel (25). The G-protein-activated effectors in turn regulate multiple downstream signaling cascades that integrate at the level of glucose metabolism, visual excitation, cardiac contractility, development and cancer (26, 27). On the other hand, the dissociated Gβγ subunits stimulate effector molecules including adenylyl cyclases, phospholipase C, and phosphoinositide 3-kinases (PI3Ks) (1) and target a range of signaling pathways involved in desensitization, apoptosis, and ion channel activation (3, 25, 28).

About 20 mammalian G protein α subunits have been identified, which can be divided into four families based on their primary sequence similarity: G_s, G_i, $G_{q/11}$, and $G_{12/13}$ (29). The signals mediated by the four G protein α subunits are named respectively: G_s pathway, G_i pathway, $G_{q/11}$ pathway, and $G_{12/13}$ pathway (30-32), as shown in Figure 1. For example, G_s couples with many extracellular signals to activate adenylyl cyclases, 12-transmembrane glucoproteins which catalyze ATP to cAMP, thereby controlling the intracellular concentrations of adenosine 3',5'-monophosphate (cAMP) (23). The cAMP produced is a second messenger in cellular metabolism and activates at least three known effectors: protein kinase A (PKA), guanine nucleotide exchange factor activated by cAMP (EPAC), and cyclic nucleotide-gated channels (33-35). PKA is an important enzyme that can regulate cell metabolism, cellular secretion, membrane permeability, and even specific gene expression (36). Activated EPAC activates the small GTPase RAP proteins, which are involved in cell growth and motility (35).

Members of the G_i family, including $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, Go, transducin ($G\alpha_t$) and gustducin ($G\alpha_{gust}$), activate a variety of phospholipases and phosphodiesterases, and promote the opening of several ion channels (37). Gi family members can inhibit a subset of these enzymes, thereby controlling the intracellular concentrations of cAMP. All isoforms of this family can be irreversibly uncoupled from their receptors by pertussis toxin (PTX). Inhibition of G_i by pretreatment with PTX causes strong impairment of lymphocyte migration *in vitro* (38), suggesting that signaling through the G_i is involved in cell motility processes.

The $G_{q/11}$ family can be divided into four subfamilies ($G_{q/11}$, G_{11}, G_{14}, $G_{15/16}$). G_q-Coupled receptors activate phospholipase Cβ, which converts phosphatidylinositol-4,5,-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol (39). These in turn lead to an increase in the

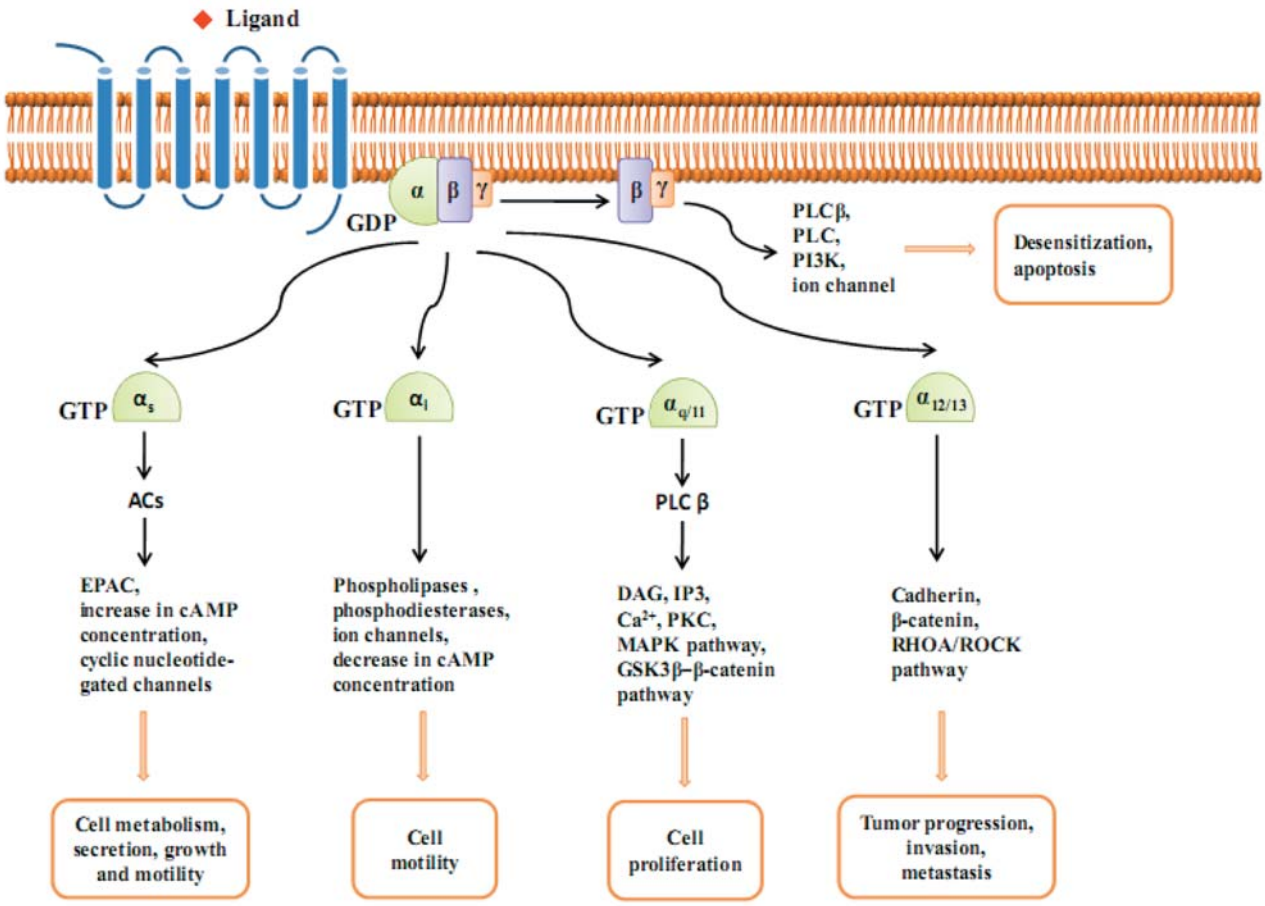

Figure 1. *G-Protein-coupled receptor (GPCR)-mediated signaling pathways. Upon activation by extracellular ligands, GPCRs can regulate key biological functions, such as cell proliferation, metabolism, secretion, motility, tumor progression, invasion, and metastasis, through signalling pathway mediated by the four G protein α subunits (G_s, G_i, $G_{q/11}$, and $G_{12/13}$).*

intracellular concentrations of free calcium and the activation of a number of protein kinases, including protein kinase C (PKC) which phosphorylates several downstream effectors such as calmodulin known to regulate calcium dependent signaling (40-43). G_q-Coupled receptors can stimulate mitogen-activated protein kinase in a PKC-dependent and Ras-independent manner depending on the cell type and the receptor expression levels (1). In addition, G_q can activate the PKC-glycogen synthase kinase-3β-β-catenin pathway, which induces cell proliferation and production of cytokines (44).

Interestingly, $G_{12/13}$ seem to be the most potent oncogenes because they comprise the only family for which overexpression of wild-type proteins has been found to be transforming (45, 46). Activation of the $G_{12/13}$ protein has been linked to the activation of small GTP-binding proteins of the RHO family, which contribute to diverse cellular processes involved in tumor progression. For example, lysophosphatidic acid-induced tumor cell migration was shown to require the activation of the $G_{12/13}$/RHOA/ROCK signaling pathway in SK-OV3 ovarian cancer cells (47). In addition, $G_{12/13}$ has been reported to interact with the cytoplasmic domain of cadherins, a family of integral membrane proteins involved in mediating cell–cell adhesion, altering their interaction with cytoplasmic proteins such as β-catenins, which suggests that $G_{12/13}$ protein mediates cancer invasion and metastasis through interaction with other cancer-related proteins (48, 49).

Classical GPCR-mediated signal transduction involves the agonist-dependent interaction of GPCRs with G proteins at the plasma membrane and the subsequent generation of soluble second messengers or ion currents by membrane localized effectors (22). However, many GPCRs directly interact with non-G protein signaling effectors, G protein-coupled receptor kinases (GRKs) and β-arrestins through specific protein–protein interaction domains (50). Active GPCRs are the target of GRKs that phosphorylate the agonist-activated form of GPCRs. This phosphorylation leads to the rapid recruitment and binding of cytosolic arrestins

Table III. *Aberrant G-protein-coupled receptor signaling in tumorigenesis and metastasis.*

GPCRs and G protein	Function	Reference
Ophan receptor		
GPR18	Tumor cell survival and metastasis	(57)
GPR48/LGR4	Invasion and metastasis	(58, 59, 65)
GPR49	Carcinogenesis and progression in cancer	(60, 61)
GPR56	Tumor cell adhesion	(62)
GPR87	Prosurvival activity	(59)
CXCR7/CMKOR1	Growth and metastasis	(59, 140)
Novel GPCRs		
GPCR-PCa	Tumorigenesis	(68)
PSGR2	Tumorigenesis	(69)
CaSR	Cancer progression and bone metastasis	(70, 72)
GPR30	Proliferation and migration	(73, 74)
GPR39	Tumorigenesis, invasiveness and metastasis	(75)
Ligands		
Sphingosine-1-phosphate	Cell growth, survival, migration, morphogenesis and blood vessel formation	(76)
Lysophosphatidic acid	Cancer initiation, progression and metastasis.	(77, 80)
Platelet-activating factor	Migration and proliferation of tumor cells and neo-angiogenesis	(141)
Thrombin	Cell proliferation and motility	(142, 143)
Interleukin 8	Angiogenesis, tumorigenicity, and metastasis	(144-148)
Growth-regulated oncogene α-γ	Tumor growth, metastasis, and angiogenesis	(1, 144, 149, 150)
Monocyte chemoattractant protein 1	Tumor growth, angiogenesis, and metastasis	(85, 151, 152)
Stromal cell-derived factor	Tumor growth, angiogenesis, and metastasis	(153, 154)

(known as arrestin-2 or β-arrestin-1, and arrestin-3 or β-arrestin-2) (51). Binding of β-arrestin proteins to the GPCRs not only uncouples the receptor from its cognate G protein, resulting in a decreased responsiveness of the signaling system to agonist (termed 'desensitization') but also initiates the process of receptor sequestration by targeting it to clathrin-coated vesicles for endocytosis (termed 'internalization') (52). Indeed, β-arrestins have been shown to act as multifunctional scaffolds and activators for a growing number of signaling proteins including ERK, p38, JNK, I-κB, AKT, and RHOA (53-55). Moreover, it has been reported that GRKs interact with a variety of proteins involved in signaling and trafficking such as $G\alpha_q$, Gβγ, PI3Kγ, clathrin, GPCR kinase-interacting protein (GIT), and caveolin (56). The characteristics discussed above highlight the complexity of the GPCR signaling pathways and the importance of considering their dynamic properties.

Aberrant GPCR Signaling in Tumorigenesis

Diverse GPCRs have been found to be overexpressed in primary and metastatic melanoma (57), human colon carcinoma (58), squamous cell carcinoma (SCC) of the lung (59), basal cell carcinoma (BCC) (60), hepatocellular carcinoma (HCC) (61), and glioblastoma multiforme (62). Some of the GPCRs and ligands reported to be involved in cancer are shown in Table III, together with their observed function. Recently, a large body of evidence has linked orphan receptors such as GPR18, GPR48/LGR4, GPR49, GPR56, GPR87, and CXCR7/CMKOR1 to the cancer phenotype. For example, Qin *et al*. found that GPR18, the most abundantly overexpressed orphan GPCR in melanoma metastasis, is constitutively active and inhibits apoptosis, indicating an important role for GPR18 in tumor cell survival (57). The links between cancer and GPR48 are somewhat more cryptic. GPR48/LGR4 is widely expressed in multiple organs (63-64), playing a vital role in development and adult physiological functions. Interestingly, Gao *et al*. found previously that up-regulation of GPR48 contributed to human colon carcinoma cell invasion and metastasis (58). Moreover, *Lgr4* knockout leads to reduced viability and retarded growth in the mouse (65). Similarly, de Lau *et al*. discovered that conditional deletion of both *Lgr4* and *Lgr5* genes in the mouse gut impairs *Wnt* target gene expression and results in the rapid demise of intestinal crypts (66), which suggested that LGR4 may be a potential target for therapy of intestinal cancer (67). GPR49 has been reported to be a novel gene marker of follicular and other tissue stem cells, overexpression of which was frequently observed in HCC with mutations in β-catenin exon 3 (61). Aberrant expression of some GPCRs also plays a role in tumor cell biology. For example, GPR56, an orphan GPCR of the secretin family, is overexpressed in gliomas and functions in tumor cell adhesion by activating the nuclear factor-kappa B, plasminogen activator inhibitor-1, and transcriptional response elements (62). Gugger and colleagues, using laser

capture microdissection and GPCR-focused Affymetrix microarrays, identified that GPR87, and CXCR7/CMKOR1 are overexpressed GPCRs in SCC of the lung (59), and could be explored as novel therapeutic targets for cancer treatment and prevention.

Recently, a series of studies showed that overexpression of some novel genes including those for GPCR-PCa, PSGR2, CaSR, GPR30, and GPR39 were associated with tumorigenesis or metastasis in diverse types of cancer tissues. In human prostate cancer, GPCR-PCa (68), which belongs to the subfamily of odorant-like orphan GPCRs, and PSGR2 (69), are selectively overexpressed and may be useful as tissue markers and molecular targets for the early detection and treatment of human prostate cancer. A previous clinical study had demonstrated that CaSR was expressed at higher levels in breast cancer cells from patients with bone metastases (70, 71). Recently, CaSR has been shown to be involved in the progression and spread of a variety of cancer types such as colorectal, breast and parathyroid, and is likely to be the focus of much research to further elucidate its precise role (72). GPR30, a seven membrane-spanning estrogen receptor, is linked to estrogen binding and heparin-bound epidermal growth factor release and induces proliferation and migration of breast cancer cells through connective tissue growth factor (73, 74). Xie *et al*. found that GPR39 was frequently overexpressed in primary esophageal SCC at both the mRNA level and protein level, which was significantly associated with lymph node metastasis and advanced TNM stage (75). In addition, many GPCR ligands, including those for sphingosine-1-phosphate (76), LPA (77-80), platelet-activating factor (81, 82), thrombin (83), interleukin-8 (84), growth regulated oncogene α-γ (1), monocyte chemoattractant protein 1 (85), and stromal cell-derived factor, have also been shown to play a key role in tumor growth, metastasis, vasculogenesis and tumor-induced angiogenesis (86). Taken together, these results suggest that interfering with these receptors and their downstream targets might provide an opportunity for the development of new strategies for cancer diagnosis, prevention and treatment (15).

High-throughput Screening (HTS) for Discovery of GPCRs Drug Targets

HTS plays a crucial role in the preclinical discovery process of many pharmaceutical companies (87). Given the importance of GPCRs as drug targets, the development of HTS for identifying target GPCRs has become a major focus in the pharmaceutical industry. Classical HTS approaches for screening GPCRs including GTP-binding assays, cAMP assays, intracellular calcium assays, inositol phosphate accumulation assays, and reporter gene assays (88). Despite the success of drug discovery aimed at GPCRs over the past decade, there remains a need to identify GPCR-targeted drugs with greater selectivity and to effectively develop screening assays for identifying lead agonists or antagonists for orphan receptors and validate these targets. In recent years, new experimental approaches, including green fluorescent protein (GFP), fluorescence resonance energy transfer (FRET), protein complementation assays (PCAs), and GPCR microarrays, have been used to analyse activation, localization, trafficking, ligand-screening assays and protein–protein interactions for GPCRs.

Tagging of GPCRs with GFP has allowed for their direct visualization of localization and real-time trafficking in living cells, which have provided crucial insight into the mechanisms involved in controlling GPCR function (89). It has been shown that GFP mutants can exhibit enhanced fluorescence properties, opening up possibilities for the development of ligand screening assays for GPCRs based on cell imaging. For instance, mutation of the tyrosine residue at position 66 to histidine generated a protein with altered spectral properties and blue fluorescence emission (90). Proof of principle following expression of a form of the β2-adrenoceptor, in which GFP was appended to the *C*-terminal tail of the GPCR (91) initiated interest in this process as a direct screening strategy. Ambrosio *et al*. have recently reviewed the use of different strategies for inserting fluorescent labels into purified, reconstituted receptors, or into receptors in intact cells to sense receptor activation *via* changes in fluorescence using modern spectroscopic and crystallographic techniques (92).

Fluorescence resonance energy transfer, a most promising approach, relies upon the dissociation of G proteins into separate α- and β/γ-subunits stimulated by GPCR activation (90). In order to study the characteristics and kinetics of GPCR activation in living cells, Milligan *et al*. have developed a strategy based on the use of FRET between donor and acceptor fluorophores attached to the receptor sequence (90). Vilardaga *et al*. initially reported this approach in 2003 (93). The principle of this assay is as follows: placement of the donor fluorescent protein CFP (27 kDa) into the third intracellular loop of the receptor and the acceptor fluorescent protein YFP (27 kDa) at the *C*-terminus results in a sensor for which conformational switches performed by the receptor after stimulation with an agonist cause an increase in the distance between the two fluorescent probes that leads to a concomitant decrease of the FRET signal. Using this strategy, parathyroid hormone and α 2 A-the adrenaline can receptor construct were generated with preserved ligand binding and signaling properties (92). Fluorescent protein-fragment complementation assay, also termed bimolecular fluorescence complementation, allow for visualization of either single or dual protein–protein interactions at the subcellular level and only require basic experimental setups (94). More recently, multicolor PCAs has been applied to quantitatively measure drug-induced

changes in GPCR interactions. For example, Przybyla and Watts used multicolor PCAs to study ligand-induced regulation and localization of cannabinoid CB1 and dopamine D2L receptor heterodimers (95). In addition, the application of PCAs-FRET combined techniques demonstrated that higher-order A2AR oligomers accumulate at the plasmamembrane in a neuronal cell model, providing insight into drug-mediated effects on GPCR signaling and oligomerization (96). With recent advances in instrumentation and the understanding of cellular mechanisms underlying the signals measured, multiplexed assays have become important tools for measuring ligand-induced receptor activation in the pharmaceutical industry. Multiplexed assays, combining reverse transfection in a 96-well plate format with a calcium flux readout, can quantitatively measure receptor activation and inhibition and permit the determination of compound potency and selectivity for entire families of GPCRs in parallel (97). However, arraying of membrane-bound proteins is complicated because they typically need to be embedded in membranes to maintain their correctly folded conformations (98). To address this problem, GPCR microarrays that require the co-immobilization of lipid molecules and the probe receptors of interest have been fabricated, using conventional robotic printing technologies (99). Using multiplexed assays by high-content imaging, Ross and colleagues identified and classified 377 compounds interfering with agonist-induced activation of the Transfluor assay, receptor internalization, or both, which indicates that the imaging assays can be used as tools to study GPCR activation and internalization (100).

Systems Biology-based Annotation for GPCR Signaling

Recently, the multidisciplinary field of systems biology has emerged as a holistic approach to interpreting the complexity and dynamics of cellular signaling (101-105). In contrast to the earlier reductionist approaches which focus on the manipulation of one gene or protein (*e.g.*, tumor suppressor or oncogene) to understand a complex entity (*e.g.*, a cell, an organ or a disease) (106), systems biology attempts to integrate global information into a comprehensive map which can be used to predict the behavior of the total system and understand how it regulates specific cells or tissues (107). Nowadays, integrative systems biology approaches have been increasingly applied to the identification of novel GPCRs and related proteins, to investigate the function of GPCRs in various disease states and to unravel GPCR complexes and signal transduction networks. Such studies should speed up the discovery of new specific drugs in cancer prevention and treatment (20). To conduct a systems-level analysis, a comprehensive set of quantitative data from 'omic' approaches is required. 'Omics' refers to the biological sciences that study the genes (genomics), their initial products (RNA transcripts) (transcriptomics), their final products (proteins) (proteomics) and the metabolites produced in the processes in which these proteins are involved (metabolomics) (108). In this review, we focus on omics-based systems biology approaches used to study GPCRs.

Genomic Profiling of Gene Expression of GPCRs

Genomics is a discipline in genetics which interrogates the molecular organization of DNA and its physical mapping, and which encompasses structural genomics and functional genomics. Advances in genomic technologies such as DNA microarrays (109, 110), and DNA chips (111) now provide an unparalleled opportunity to perform large–scale global analyses of the variability of gene expression. DNA arrays can be used for many different purposes, most prominently to measure levels of gene expression (messenger RNA abundance) for tens of thousands of genes across different tissues or cells simultaneously (109). Several groups have now taken advantage of this technology to perform global analyses of the variability of gene expression between normal tissue and abnormal tissue, especially in tumor tissue. For example, Ye *et al*. used 15 genomic expression arrays, each of which included 223 GPCR transcripts presented in at least 1 out of 15 of the independent microarrays, to analyze samples from normal adrenals, aldosterone-producing adenomas and cortisol-producing adenomas (112). The array results indicated that certain GPCRs exhibit elevated expression in the former. These data were further confirmed using real-time RT-PCR (qPCR), which suggested a potential role for elevated expression of GPCR in many cases of primary hyperaldosteronism and provided candidate GPCRs for further clinical study. Lockhart and Winzeler used high-resolution oligonucleotide comparative genomic hybridization (CGH) arrays to match gene expression array data and identified dysregulated genes that were able to classify breast cancer according to gene copy number anomalies (109). This study first showed that CGH arrays were a robust technology for assessing gene copy number anomalies and provided a new strategy to screen novel therapeutic targets for molecular subsets of cancer.

An understanding of mutations and alterations in the expression of various genes resulting in carcinogenesis combined with the development of microarray technology has enabled the identification of comprehensive gene expression alterations during oncogenesis (113-116). In recent years, many studies have applied this technology for BCC (60), HCC (113) and other type of cancer and have identified a number of candidate genes with potential as biomarkers in cancer staging, prediction of recurrence and prognosis, and treatment selection. Interestingly, Tanese *et*

al. screened a DNA microarray database of human BCC cases to identify genes responsible for tumor formation in BCC and found up-regulation of the orphan GPCR GPR49 in all BCC cases compared with controls, which was confirmed by real-time qPCR analysis and *in situ* hybridization (60). These data suggested that GPR49 may play a vital role in tumor formation. Similarly, Weigle *et al*. screened a DNA Chip-based expression database and identified an expressed sequence tag (EST) originally sequenced in a lung cancer cDNA library for the identification of target structures specifically expressed in prostate tissue (68). They identified GPCR-PCa as a novel putative GPCR that was overexpressed in prostate cancer.

Transcriptomics Profiling of GPCR Expression

Transciptomics (or expression profiling) describes the complete set of RNA transcripts present in a particular cell including messenger RNA (mRNA), micro-RNA (miRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA) (117). Unlike the genome, which is fixed for a given cell type (excluding mutations), the transcriptome can be modulated by external environmental conditions. Nowadays, a number of techniques are available for studying transcriptomics, including cDNA hybridization microarrays, serial analysis of gene expression (SAGE), conventional RT-PCR and qPCR.

Using qPCR, Maurel *et al*. identified a number of GPCRs that had not been detected previously in cerebellar granule neurons and neuroblasts during postnatal development (118). These GPCRs represent novel candidates in the development and survival of cerebellar granule neurons. Using cDNA microarray analysis, Xie and co-workers identified a GPCR, GPR39, which is significantly up-regulated in esophageal SCC. They then further analyzed the mRNA expression level of GPR39 in 9 esophageal SCC cell lines and 50 primary esophageal SCC tumors using semi-quantitative RT-PCR (75). Interestingly, functional studies *in vitro* and *in vivo* showed that GPR39 plays an important tumorigenic role in the development and progression of esophageal SCC. Atwood *et al*. using microarray analysis revealed expression of GPCRs and related proteins in HEK293, AtT20, BV2, and N18 cell lines, providing a better understanding of the potential interactions between GPCRs and these signaling proteins (119). In addition to mRNA, ESTs can also give valuable information about expression patterns. Fredriksson and Schioth identified more than 20,000 sequences that match GPCRs through searching EST databases (120). GPCRs receptors are encoded by low abundance mRNAs and can be fully functional at levels of 1×10^4 protein molecules per cell (121). It can therefore be difficult to determine the expression profiles of GPCRs in cell lines or in tissues using conventional cDNA or oligonucleotide arrays. Thus there is a need to develop new methodologies to apply to the GPCR genome. The use of microarray technology allows for a large sampling of receptor families with low abundance mRNAs to be performed and also allows monitoring of the message levels of thousands of genes simultaneously in a given sample (122, 123). Hansen *et al*. developed a new method based on multiplex PCR and array detection of amplicons to assay GPCR gene expression using as little as 1 μg of total RNA. Using this method, they profiled three human bone marrow stromal cell lines (124). Hakak and co-workers used a custom high-density oligonucleotide microarray containing probes designed to measure the gene expression levels of over 700 human GPCRs, along with a number of molecules involved in GPCR signaling and regulation (125). These studies revealed complex signaling networks in many cell types.

Evaluation of the transcriptional levels for these genes across a large panel of tissues would thus provide a global view of GPCR expression and function in the human body (125). Yamamoto and colleagues have investigated the differences in mRNA expression in several HCC cell lines by using mRNA differential display polymerase chain reaction (mRNADD-PCR) (61). This study demonstrated that GPR49, which belongs to the glycoprotein hormone receptor subfamily, is markedly up-regulated in HCCs carrying β-catenin mutations. To better understand the functions of GPCRs *in vivo*, Regard *et al*., by analyzing the pattern of GPCR mRNA expression across tissues and the relative abundance for each of 353 nonodorant GPCRs in 41 tissues from adult mice, developed a dataset that provides a useful resource for finding previously unidentified roles for GPCRs with known ligands, and importantly provides clues regarding the function of orphan GPCRs and the source of their ligands (126). Additionally, they demonstrated that the GPR91, a receptor for the citric acid cycle intermediate succinate, can regulate lipolysis in white adipose tissue, suggesting that signaling by this citric acid cycle intermediate may regulate energy homeostasis.

Proteomics Identification of Novel GPCRs and Their Interactive Proteins

Proteomics is the large-scale analysis of proteins to profile a whole proteome or sub-proteome in a single experiment, so that the protein alterations corresponding to a pathological or biochemical condition at a given time can be annotated in an integrated way (127). Proteomics can be divided into three main areas: protein micro-characterization for large-scale identification of proteins and their post-translational modifications; 'differential display' proteomics for comparison of protein levels with potential application in a wide range of diseases; and studies of protein–protein interactions using techniques such as mass spectrometry (MS) or the yeast two-hybrid system to give information on signaling pathways (74).

Nowadays, proteomics strategies encompass many analytical techniques including high resolution two-dimensional electrophoresis (2-DE), multidimensional separation protocols for the purification of trace components (128), two-dimensional difference gel electrophoresis (2D-DIGE), antibody/protein arrays, metabolic or chemical labeling techniques such as stable isotope labeling by amino acids in cell culture (SILAC), gel-free systems such as isotope-coded affinity tagging (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), proteolytic ^{18}O labeling, coupled with advances in MS such as multidimensional protein identification technology (MudPIT) and multiple-reaction monitoring (MRM) (129), which combine to provide the large–scale and unbiased platforms required to determine the dynamic profiles of GPCR proteomes.

Proteomics offers specific advantages for the analysis of GPCR complexes and signal transduction networks. Because these regulatory mechanisms are not necessarily dependent on *de novo* synthesis, genomic and transcriptomic approaches might fail to identify these processes (130). During the past decade, MS analysis has gained prominence for revealing detailed information on the individual GPCR class, including characterization of the GPCR binding pocket and specific post-translational modifications. For example, using MALDI-TOF and/or LC-ion trap MS, Kamonchanok *et al*. were able to directly determine >80% of the primary amino acid composition of the histamine H1 receptor covering five of the transmembrane domain regions after baculovirus-driven and *in vitro* cell-free expression (131).

GPCRs are associated with large protein networks organised by protein–protein interactions involving multidomain proteins. The combination of 2-DE or high-resolution chromatography and MS allows more direct observations to be made based on the study of molecular interaction networks. Using the *C*-terminal tail of the 5-hydroxytryptamine 2C (5-HT2C) receptor as an example, Bécamel and co-workers identified at least 15 proteins that interact with the *C*-terminal tail of the 5-HT2C receptor using a proteomics approach based on peptide affinity chromatography followed by MS and immunoblotting. These studies indicated that the 5-HT2C receptor is associated with protein networks that are important for synaptic localization and coupling to the signaling machinery (132). β-Arrestins are cytosolic proteins that form complexes with seven-transmembrane receptors after agonist stimulation and phosphorylation by GPCR kinases. Xiao and co-workers performed a global proteomics analysis of β-arrestin-interacting proteins (interactome) as modulated by a model seven-transmembrane receptor, the angiotensin II type 1α receptor, in an attempt to assess the full range of functions of these versatile molecules. In this study, they combined gel-based and non-gel-based proteomics methods in order to enhance the coverage and reliability of the analysis and identified 337 nonredundant proteins interacting with β-arrestin 1 and 2 using nano-LC MS/MS (133). These proteins, which were ubiquitously distributed in the cell, had multiple functions, including receptor desensitization, endocytosis, signal transduction, regulation of gene expression, protein synthesis, cellular reorganization, chemotaxis, and apoptosis.

Protein phosphorylation is a general and important mechanism of cellular regulation that involves at least two interacting protein partners (134, 135). In recent years, global and site-specific analysis of *in vivo* phosphorylation sites by quantitative MS has emerged as the method of choice to investigate cell signaling pathways in an unbiased fashion (136). For instance, using SILAC in combination with specific phosphopeptide enrichment using TiO2 chromatography (135) and high performance MS using a LTQ-Orbitrap MS, Christensen *et al*. compared the phosphoproteomes of the AT1R agonist angiotensin II and the biased agonist [Sar^1, Ile^4, Ile^8] angiotensin II (SII angiotensin II). As a result, they quantified more than 10,000 phosphorylation sites of which 1183 were regulated by angiotensin II or its analog SII angiotensin II (137).

Conclusion

The GPCR superfamily is one of the largest families of proteins in mammalian genomes, whose primary function is to transduce extracellular stimuli into intracellular signals regulating a host of physiological and disease processes. Although GPCRs represent an important group of targets for pharmaceutical therapeutics, difficulties in the identification of their natural ligands has impeded development of therapeutics based on these potential drug targets. Breakthrough in high-throughput screening technologies has helped solve this. Nowadays, integrative systems biology approaches are being increasingly applied to GPCRs to unravel the biological mechanisms of human diseases, especially cancer, and to overcome the limitations faced in diagnosis and drug development. At present, the challenge for systems biology is to effectively and efficiently integrate multidisciplinary approaches, including engineering, computational analysis, and physics with biological and medical inputs in order to understand the complex dynamic network of interactions within a cell that regulate cellular, organ, and organism behavior (138). Towards this end, it is necessary to develop common platforms for the analysis, formatting and archiving of data to ensure inter-laboratory and cross-disciplinary compatibility and accessibility of datasets (139). With increasing challenges and novel questions emerging, new technologies and methodologies urgently need to be developed. New systems biology approaches that can accurately quantify predetermined sets of molecules (proteins, phosphoproteins, metabolites, lipids) at very high sensitivity, reproducibility, and wide dynamic range

seem to better deal with systems biology's hunger for high-quality datasets. With the development of high throughput technologies with the potential to generate unprecedented amounts of dynamic signaling data, the challenges now lie in more theoretical and conceptual areas. State of the art bioinformatics and mathematics will be developed to handle the complexity associated with the extensive signaling networks associated with GPCR biology (20). These emerging technologies and methodologies will help to reveal the potential utility of this understanding for overcoming the aberrant signaling associated with disease states.

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