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OLD RECEPTORS LEARN NEW TRICKS: BIASING ANTI-IGF1R CANCER THERAPY THROUGH THE GPCR SYSTEM

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Old receptors learn new tricks: Biasing anti-IGF1R cancer therapy through the GPCR system

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my famlly

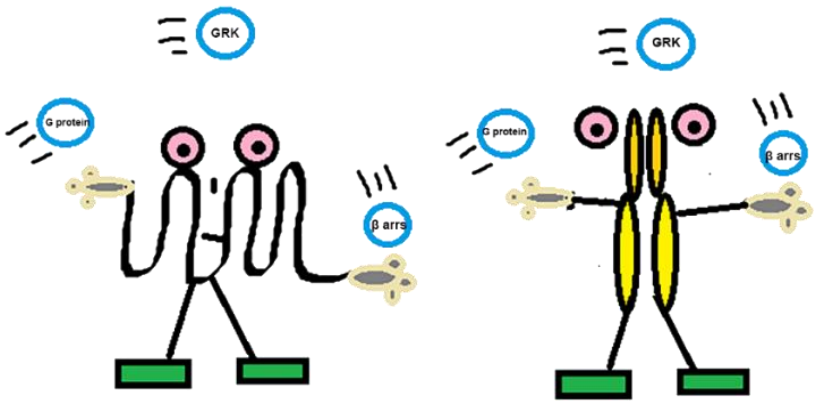
致我的家人

POPULAR SCIENCE SUMMARY OF THE THESIS

The cells in our body are brilliant enough to self-repair when facing challenges throughout their lifetime. However, sticking to the right repair program is not always the case; more specifically, the chance is like winning a lottery. Every repair, cells draw once from their "lotto pool" to win the first prize of immortality, which means the winner becomes a potential cancer cell. Cancer risk factors, like smoking, drinking alcohol, allow cells to draw the lotto more frequently, hence resulting in a higher chance of cancer if they can escape from immunological surveillance and find suitable soil to grow.

Then how can these potential cancer cells gain the ability of unrestricted proliferation? Cellular signals play a critical role in this process. Most cells receive messages from their surroundings; When the messages are delivered, receptors on the cancer cells can receive and translate these signals into their specific function. If the receptor does not function appropriately, for example by being blocked, the cell cannot receive signals and may not survive. Due to this fact, many companies and researchers designed and developed many receptor-targeting drugs to treat cancers. The main study object in my PhD program, the insulin growth factor 1 receptor (IGF1R), is an essential receptor regulating cancer progress.

IGF1R inhibitor agents have been applied in cancer therapy for many decades, due to their strong potential to kill the tumor, in theory. Nevertheless, accumulated clinical outcomes fail to reach that expectation, because tumor reoccurrence is often seen after this treatment. Many researchers believe that IGF1R is still a strong candidate in cancer treatment. However, how to effectively block it, remains unclear. In recent years, the relationship between IGF1R and GPCRs (G protein-coupled receptors) has attracted attention of researchers. In my study, I have disclosed how IGF1R controls GPCR partners and vice versa.



"Old receptors learn new tricks"

Unlike IGF1R, GPCRs are another family of receptors controlling numerous physiological and pathological processes. Various GPCRs targeting agents have already been approved by the FDA. Still, very few of them are proposed for cancer therapy. It has been demonstrated that some of GPCR targeting drugs can improve the efficiency of IGF1R-targeting treatment. In light of our recent findings, anti-IGF1R cancer treatment via regulating the GPCR system, may provide promising therapeutic strategies for the clinic.

Altogether, this thesis aims to find the possibility of using drugs that target GPCR modulators to block IGF1R signaling in cancer treatment. Let the old "joker" learn new tricks.

ABSTRACT

As cancers progress, tumor cells exploit the extracellular signals generated from plasma membrane receptors for cell growth, migration, and anti-apoptosis. G protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTKs) are two important families of plasm membrane receptors, controlling multiple biological functions via their downstream signaling. IGF1R, one of the major RTKs involved in developing the malignant phenotype, plays a critical role in the tumorigenesis of multiple cancers. Thus, anti-IGF1R antibodies and inhibitors soon became attractive stars in cancer treatment. Disappointingly, IGF1R lost its "glory" in clinical trials with a promising start but no happy end. However, lessons from those clinical trials led us to explore the underlying mechanisms behind anti-IGF1R cancer therapy. One of the outcomes is that IGF1R interacts with GPCR downstream modulators (G proteins, GRKs, β-arrestins), which are vital in coordinating IGF1R downstream signaling. This thesis aims to refine the concept of IGF1R targeting through GPCR components and translate it into clinical application.

In **Study 1**, we investigated the potential therapeutic mechanisms of the IGF1R/ β-arrestin /p53 axis in conjunctival melanoma (CM). This research revealed the targeted therapeutic strategy of controlling IGF1R and p53 pro/suppressor tumorigenic signals via β-arrestin1/MDM2, thus reducing tumor growth and the risk of metastasis. In **Study 2**, we studied the molecular mechanism of inhibiting IGF1R through "system bias". Our work highlights unbiased downregulation of IGF1R via GRK2 inhibition in Ewing's sarcoma. These findings reveal the molecular and biological roles of biased signaling downstream of IGF1R and its potential therapeutic application in clinical settings. In **Study 3**, we investigated the therapeutic strategies of targeting IGF1R via "system bias" in colorectal cancer. This work demonstrated that paroxetine (PX) could downregulate both IGF1R and the epidermal growth factor receptor (EGFR), resulting in inhibition of cancer cell viability. When combining PX with MAPK and PI3K inhibitors, the combination treatment showed an additive inhibition effect on tumor growth and metastasis. This study revealed a strategy of controlling signaling pathways residual to system bias inhibition. In **Study 4**, we studied the involvement of G proteins in the IGF1R system. We revealed that G protein signaling regulates IGF-induced cell growth in both in vivo and in vitro experiments and their inhibition induces receptor internalization via the GRK/β-arrestin system. This study expanded the RTK-GPCR dualism paradigm of the IGF1R and explored the concept of G-protein signaling targeting in cancer.

To summarize, our studies highlight the potential of targeting IGF1R via the GRK/β-arrestin system and suggest the possibility of clinical translation of this novel concept into different

cancer types. These findings broaden our understanding of the IGF1R system and open a brand-new chapter in anti-IGF1R cancer therapy.

LIST OF SCIENTIFIC PAPERS

I. Crudden C, Shibano T, **Song D**, Dragomir MP, Cismas S, Serly J, Nedelcu D, Fuentes-Mattei E, Tica A, Calin GA, Girnita A, Girnita L.
Inhibition of G Protein-Coupled Receptor Kinase 2 Promotes Unbiased Downregulation of IGF1 Receptor and Restrains Malignant Cell Growth. Cancer Res. 2021 Jan 15;81(2):501-514; doi: 10.1158/0008-5472.CAN-20-1662

II. **Song D**, Cismas S, Crudden C, Trocme E, Worrall C, Suleymanova N, Lin T, Zheng H, Seregard S, Girnita A, Girnita L.
IGF1R is a molecular determinant for response to p53 reactivation therapy in conjunctival melanoma. Oncogene. 2021 Nov 17. doi: 10.1038/s41388-021-02111-x.

III. **Song D**, Takashi S, Wang X, Pasca S, Zhang S, Cismas S,Crudden C, Girnita A, Calin GA, Girnita L.
G protein-coupled receptor kinase 2/β-arrestin-1 system bias inhibition of IGF1R/EGFR induces sensitization of the InsR receptor and destabilizes the metastatic phenotype of colorectal carcinoma. Manuscript.

IV. **Song D**, Cismas S, Pasca S, Zhang S, Wang X, Crudden C, Takashi S, Girnita A, Calin GA, Girnita L.
G protein activation balance the signaling pathways downstream insulin-like growth factor 1 receptor: therapeutic implications for cancer. Manuscript.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THESIS

1.Crudden C, **Song D**, Cismas S, Trocmé E, Pasca S, Calin GA, Girnita A, Girnita L.

Below the Surface: IGF1R Therapeutic Targeting and Its Endocytic Journey. Cells. 2019 Oct 9;8(10):1223.

2. Crudden C, Shibano T, **Song D**, Suleymanova N, Girnita A, Girnita L.

Blurring Boundaries: Receptor Tyrosine Kinases as functional G Protein-Coupled Receptors. Int Rev Cell Mol Biol. 2018; 339:1-40.

CONTENTS

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
GPCR	G protein-coupled receptor
IGF1R	Insulin-like growth factor type 1 receptor
SH2	Src Homology 2
TM	Transmembrane
G protein	Guanine-nucleotide-binding regulatory proteins
IGFs	Insulin-like growth factors
IGF1	Insulin-like growth factor 1
IGF2	Insulin-like growth factor 2
IGFBPs	IGF-binding proteins
IGF2R	Insulin-like growth factor type 2 receptor
IRR	Insulin related receptor
AMP	Adenosine monophosphate
GRKs	G protein-coupled receptor kinase
RH	RGS homology
PH	Pleckstrin homology
β2AR	β2-adrenergic receptor
RTK	Receptor tyrosine kinase
pIGF-1R	Phosphorylated IGF-1R
pAkt	Phosphorylated Akt
pERK	Phosphorylated ERK
C-terminal	Carboxyl-termina
EGFR	Epidermal growth factor receptors (EGFR, ErbB and HER)
PDGFR	Platelet-derived growth factor receptor
FGFR	Fibroblast growth factor receptor
VEGFR	Vascular endothelial growth factor receptor
ALK	Anaplastic lymphoma kinase
IR	Insulin receptor
CM	Conjunctival melanoma
PX	Paroxetine
Mdm2	Mouse double minute 2 homolog
NEDD4	Neuronal precursor cell-expressed developmentally downregulated 4
CBL	Casitas B-lineage Lymphoma
HRD1	HMG-CoA reductase degradation protein 1
MAPK	Mitogen activated protein kinase

ERK1/2	Extracellular signal-regulated kinases
MEK	Mitogen-activated protein kinase kinase
PI3K	Phosphatidylinositol 3-kinase
Akt (PKB)	Protein kinase B
PKA	Protein kinase A
PKC	Protein kinase C
FDA	Food and Drug Administration
ECL	Extracellular loops
ICL	Intracellular loops
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
AC	Adenylyl cyclase
DAG	Diacylglycerol
RhoGEF	Rho guanine nucleotide exchange factor
AMP	Adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
PLCβ	Phospholipase C β
CREB	cAMP response element-binding protein
JNK	Jun N-terminal Kinase
α1AR	α1 adrenergic receptor
AT1R	Angiotensin II type 1 receptor
CXCR4	C-X-C chemokine receptor 4
Shc	Src homology 2-containing protein
IRS1	Insulin receptor substrate 1
FRS2	Fibroblast Growth Factor Receptor Substrate 2
PLCγ	Phospholipase C γ
NPXY	Asn-Pro-x-Tyr
A-loop	Activation loop
Grb2	Growth factor receptor-binding protein 2
SOS	Son of sevenless
mTOR	Mammalian target of rapamycin
PIP2	Phopsphatidyl-inositol bisphosphate
PIP3	Phopsphatidyl-inositol trisphosphate
PDK1	3-phosphoinositide-dependent protein kinase 1
Bad	Bcl-2-associated death promoter
FOXO	Forkhead box O

Bcl-2	B-cell lymphoma 2
GSK-3β	Glycogen synthase kinase 3β
PPP	Picropodophyllin
MEF	Mouse embryonic fibroblast
LDS	lithium dodecyl sulphate
IHC	Immunohistochemistry
WB	Western blot
TBS	Tris-buffer saline
PVS	Perivitelline space

1 INTRODUCTION

1.1 CELL SIGNALING AND CANCER

Cells use receptors to convert external stimuli into cellular signals; such intracellular signals are transmitted to the nucleus or other subcellular compartments and further converted into biological responses. These processes are essential for controlling homeostasis and maintaining the normal function of the cells. On the other hand, the dysregulation of cell signaling can break homeostatic states, thus contributing to several diseases, including cancer (*1*). Cancer is a group of diseases with many deranged pathways, such as replicative immortality or deregulated metabolism, commonly known as hallmarks (*2*). However, among the top ten cancer hallmarks, dysregulated cell signaling is probably the only one critically involved in regulation of all the other nine cancer traits (Fig.1).

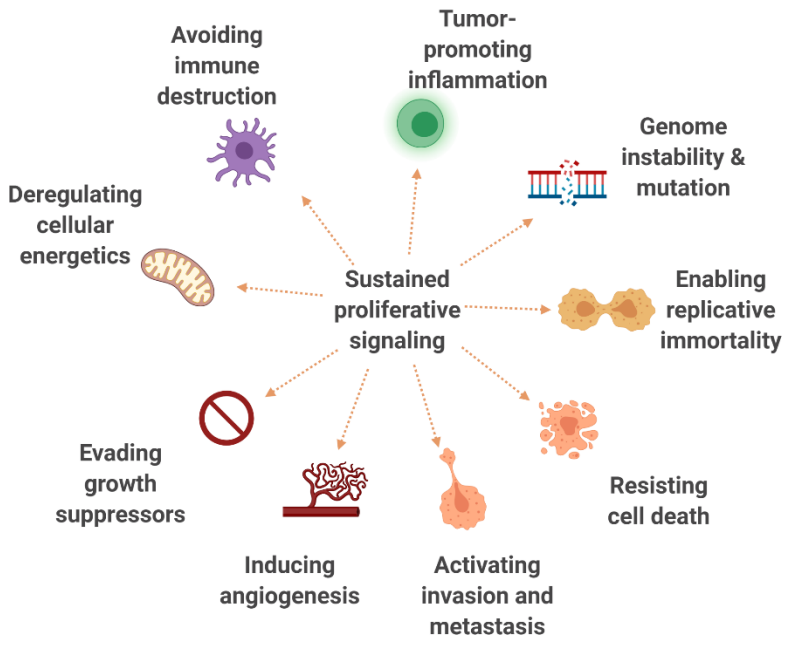


Figure 1. The relationship between cell signaling and other hallmarks of cancer.

As the critical structures in receiving and transferring cellular signals, receptors crossing the plasma membrane play crucial roles in mediating information transfer within a multicellular organism. The receptor receives the message from its extracellular space via ligand binding. Then signaling elements interact with transcription factors and translate the message into biological activities (*3*). Most transmembrane receptors have their own specific ligands, that mediate their particular functions. They can be classified into four groups: G protein-coupled receptors (GPCRs), enzyme-linked receptors (RTK belongs to this family), ligand-gated ion

channels, and nuclear receptors. This literature review will focus on two of them: GPCRs and RTKs.

1.2 GPCR SYSTEM

The GPCR family is the largest family of plasma membrane receptors which consists of more than 800 different receptors (*4*). GPCRs mediate signaling that control important physiological and pathophysiological functions. Owing to their involvement in essential biological processes and easy access at the cell surface, GPCRs have become essential therapeutic targets for a large majority of human diseases. More than 30% of all drugs approved by the FDA work though targeting GPCR or their downstream signaling pathways (*4-6*).

All GPCRs have a comparable structure consisting of an N-terminal extracellular domain, seven transmembrane (TM) domains which are linked by three extracellular loops (ECL), three intracellular loops (ICL) and a C-terminal tail on the intracellular side of the membrane (*7, 8*). The polypeptides of GPCRs thread back and forth across the plasma membrane seven times and form a ligand-binding site within the receptor's central part (*9*). Several signal molecules can bind to GPCRs and activate its downstream signals, such as small peptides, proteins, amino acids, hormones, and lipids. Three classes of protein control the expression and function of GPCRs: G-proteins, GRKs and β-arrestins (*10, 11*).

1.2.1 G protein

The human heterotrimeric G protein family consists of about 20 alpha(α) subunits, 5 beta (β) subunits and 12 gamma (γ) subunits. The G protein α-subunit can be further classified into 4 families, Gαs family, Gαi family, Gαq/11 family, and Gα12/13 family according to their functions: Gαs(stimulatory) family is the first discovered G protein family (*12*). Gαs-GTP (Active Gαs) can bind to and stimulate adenylyl cyclase (AC), increase cyclic adenosine monophosphate (cAMP) production; Gαi (inhibitory) can also directly bind to AC, but it has an opposite function comparing with Gαs. Gαi inhibits AC activity and decreases the level of cAMP in the cells (*13, 14*). Gαq/11 family controls the activation of PLCβ downstream of GPCR signaling, resulting in Ca^{2+} release via Ins (1,4,5) P3 and protein kinase C (PKC) activation via diacylglycerol (DAG) (*15*); Gα12/13 family is responsible for activation of p115RhoGEF (*16, 17*). Gβγ subunits activities depend on the particular GPCR being activated (*18*). Following the G protein complex dissociation, Gβγ interacts with its partners to induce

signal transduction (*19, 20*). In addition, free Gβγ subunits in the cytoplasm recruit GRK2 to the activated receptor, which is an essential step in GPCR desensitization (*21*).

G protein	Isoform	Function	Inhibitor
$G\alpha_s$	$G\alpha s_{short}$ / $G\alpha s_{long}$ /$G\alpha_{olf}$	Adenylyl cyclase (+) cAMP (+)	Suramin (*22*)
$G\alpha_i$	$G\alpha_o$, Gαi1, Gαi2, Gαi3, $G\alpha_z$, $G\alpha_g$ and Gαt	Adenylyl cyclase (-) cAMP (-)	Pertussis toxin (*23*)
$G\alpha_{q/11}$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$ ($G\alpha_{15}$ in mouse)	PLCβ activation Release of Ca^{2+}	YM-254890 (*24*) FR900359 (*25*) BIM-46187 (*26*)
$G\alpha_{12/13}$	$G\alpha_{12}$ and $G\alpha_{13}$	p115RhoGEF and Rho activation	
Gβγ	5 Gβ subunits 12 Gγ subunits	GRK recruitment Signal transducer	βARKct (GRK2ct) (*27*) Gallein (*28*)

Table 1. G proteins family classification, isoforms, function and inhibitors.

1.2.2 GRK/β-arrestin system

G protein receptor kinases (GRKs) are ubiquitously expressed protein kinases which belongs to the AGC kinase family (*29*). There are seven GRK isoforms (GRK1–7) encoded in the human genome (*30*). They can be divided into three families based on their structural resemblance: visual GRKs (GRK1/7) (*31, 32*), β-adrenergic receptor (β2AR) kinases (GRK2/3), and the GRK4 family (GRK4/5/6) (*33*). The visual GRK family is only expressed in retinal rod and cone cells. The GRK4 family contains isoforms 4, 5, and 6 which directly use their PIP2 binding sites near the N-terminus to anchor at the plasma membrane (*34*). GRK5 and GRK6 are universally expressed in human tissues, yet GRK4 is only expressed in testis tissue. The β-adrenergic receptor kinase family contains GRK2 and GRK3, they share a pleckstrin homology (PH) domain in their c-terminus for Gβγ binding and these kinases are recruited from cytoplasm to the plasma membrane (*35, 36*). GRKs can recognize GPCR activation, phosphorylate serine residues on the intercellular loops and c-terminus of the receptor, and then recruit β-arrestin (*37-39*).

There are four different isoforms in the arrestin family: visual arrestins (arrestin 1 and arrestin 4) are exclusively found in the retina (*40, 41*). In contrast, arrestin 2 and 3 (β-arrestin 1 and 2) are expressed ubiquitously in all tissues (*42, 43*). β-arrestin 1 and 2 share a similar structure and regulate most GPCRs. β-arrestins can find and bind to GRK-phosphorylated sites on the receptor, which characterized major mechanism of receptor desensitization. The main functions of GRK/β-arrestins system are: 1) To prevent G proteins from coupling to the activated receptor (*44, 45*); 2) To internalize the receptor via clathrin-mediated endocytosis (*46*); resulting in desensitization of the receptor and "turning off" the signal (*45, 47, 48*).

1.2.3 GPCR signaling

1.2.3.1 G protein signaling

The binding of the ligand to the GPCR stabilizes the active conformation of the receptor. The receptor then recruits the G protein and exchange the Guanosine diphosphate (GDP) on the Gα subunit for the Guanosine triphosphate (GTP) exchange. Gα subunit activation results in the disassociation of Gβγ from the G protein heterotrimeric complex (*49*). G protein signaling promotes the formation of second messengers such as cAMP, Phospholipase C β (PLCβ) (*50*), Rho guanine nucleotide exchange factors (RhoGEF) (*51*) and diacylglycerol (DAG) (*52*), which lead to various biological effects (*9, 53*). For instance, cAMP is at the central position of controlling several signaling pathway (*54*). One of the main functions of cAMP is to active protein kinase A (PKA) pathway: cAMP induced PKA conformation changes that release catalytic (C) subunits. After, PKA-cat goes into the nucleus then phosphorylates the transcription factors such as CREB (*55, 56*); which mediates various physiological processes including exocytosis (*56*), cardiovascular function (*57*) and memory (*58, 59*).

1.2.3.2 β-arrestin signaling

Despite their canonical role of desensitizing GPCRs, it has been shown that β-arrestins are independent signal transducers, that can couple to the receptors and act as adapters or scaffolds. β-arrestins have the ability to scaffold with signal modulators, resulting in a second wave of GPCR signaling, G protein independent, β-arrestin signaling (*60-62*).However, recent studies also suggest dependency of β-arrestin signaling on G proteins (*63*). β-arrestins can initiate MAPK and PI3K-AKT signaling by binding to key signal molecules like c-Src (*64*) , JNK3 (*65, 66*), MEK (*65, 66*), AKT (*67, 68*), PI3K (*69, 70*). Altogether, it is clear that β-arrestin signaling plays an essential role in regulation of GPCR signaling.

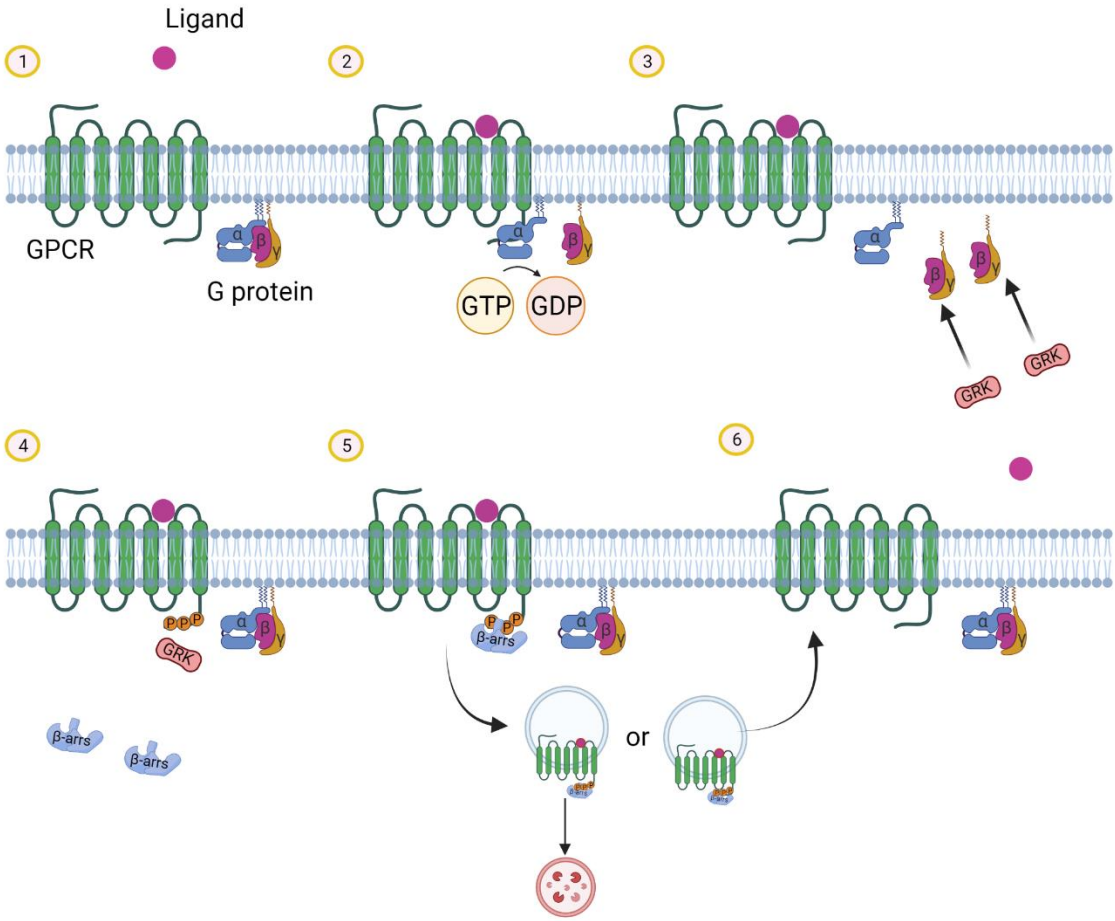


Figure 2. Regulation of GPCR trafficking. (1). The inactive state of GPCRs. (2). Ligand binding triggers the GTP exchange from GDP on Gα subunits. (3). Dissociated Gβγ subunits recruit GRKs. (4). GRKs phosphorylate intercellular loop of GPCR resulting in β-arrestin recruitment. (5-6). β-arrestins block G protein coupling and then trigger receptor internalization. GPCRs will either be degraded or brought back to the cell surface via recycling.

1.2.4 GPCR trafficking

Activated receptors are phosphorylated by GRKs, which increase the recruitment of the multifunctional proteins, β-arrestins to the receptor; then β-arrestins bind to the receptor and interact with clathrin-coated pits to induce signals controlling endocytosis. Afterward, the GPCR/β-arrestin complex in the cytoplasm will be either recycled or degraded through the lysosome and proteasome system (*47, 71*). According to the affinities of β-arrestin to the GPCR, GPCRs can be classified into two subgroups: Class A receptors (e.g. β2AR, α1AR), which have a higher affinity to β-arrestin 2, bind to the receptor transiently and undergo recycling; by contrast, Class B receptors (e.g. AT1R, vasopressin V2 receptor) show equal affinity to β-arrestin1 and 2, bind stably and result in receptor degradation (*72, 73*).

As a negative feedback mechanism, β-arrestins cease the G protein signaling and remove the receptor from the cell surface (*48, 74, 75*). The internalized receptors can be degraded through the proteasomal and lysosomal degradation system or brought back to the plasma membrane via recycling (*76*). The whole process is illustrated in Figure 2.

1.2.5 Biased signaling

A major cell biology dogma is that ligand-receptor binding is essential for signaling activation. Upon ligand binding, GPCR switches from an inactive state into an active state, leading to balanced activation of both G protein and β-arrestin signals. However, studies from a few decades ago challenged this theory, showing the existence of non-balanced agonists or antagonists that can preferentially activate either the G protein signal or β-arrestin signal and produce particular functional outcomes (*77, 78*).

Unbalanced signal activation (biased signaling or functional specificity) can be modified from the extracellular side (biased ligand), intracellular adaptors (biased system) and also the receptor itself (biased receptor). A biased ligand can induce receptor-specific conformational change that increases the affinity to a particular signal transducer. This interaction results in activation of certain pathways and inhibition of other pathways, e.g. G protein signal increasing while β-arrestin signal decreasing. The other way that has been well-established is that a GPCR signal can be biased via allosteric modification from the intracellular surface, which is so-called "system bias" (*77*). System bias can be induced by the GRK/β-arrestin system, by different levels of their expression or activity. Some cell-permeable peptides, like pediocins, also show abilities to bias receptor signaling as allosteric modulators. For examples ATI-2341 selectively activate Gi, which promotes Gi biased signaling downstream of CXCR4; the intracellular loop (ICL) 3-9 of β2AR enhances Gs signal while ICL1-9 induces β-arrestin biased signal (*79*).

The development of biased ligands has become an attractive area in pharmacological research. Some biased drugs have already been used to treat for example heart failure, pain management, asthma, Parkinson's disease, sclerosis. Interestingly, G protein signaling and β-arrestin signaling downstream of one GPCR can show different functional roles (*80*). At AT1R, G protein signaling is related to vasoconstriction and cardiac hypertrophy, contributing to heart failure (*81*). However, the β-arrestin signaling downstream of AT1R ceases the G protein signals and increases intracellular calcium concentration and anti-apoptotic signals, resulting in cardioprotective functions (*82, 83*). Based on these findings, β-arrestin biased agonists or antagonists of AT1R were designed, such as SII angiotensin, TRV027 which FDA already approved for clinical use (*84, 85*).

1.3 RTK

Receptor tyrosine kinases (RTKs) are the second largest family among all receptor families. There are at least 58 RTKs in human genome and they are in charge of diverse biological functions. RTKs have a similar structure which consists of a ligand-binding domain (extracellular) at the N-terminus, a transmembrane domain, and a tyrosine kinase domain (intercellular) at the C- terminus. Most of the known RTKs are expressed as a monomer when inactivated (Figure 2, 1). Their respective ligand binds to the ligand-binding domain of inactivated RTKs, bringing two monomers together to become a dimer (receptor dimerization) (Figure 2, 2). This is followed by cross phosphorylation of these two cytoplasm kinase domains, promoting kinase activation of each other (Figure 2, 3). Interestingly, unlike other RTKs, the insulin receptor and IGF-1 receptor have already performed as a dimer; the mechanism of this family will be discussed in detail later. Kinase phosphorylation on the intercellular domain generates docking sites for signal transducers and effectors such as IRS1, FRS2, PLCγ, and Shc (Figure 2, 4) (*86*, *87*). Subsequently, phosphorylated receptors can use molecules to activate signals through different signaling pathways. There are two well-studied RTK downstream cascades: MAPK and PI3K-AKT.

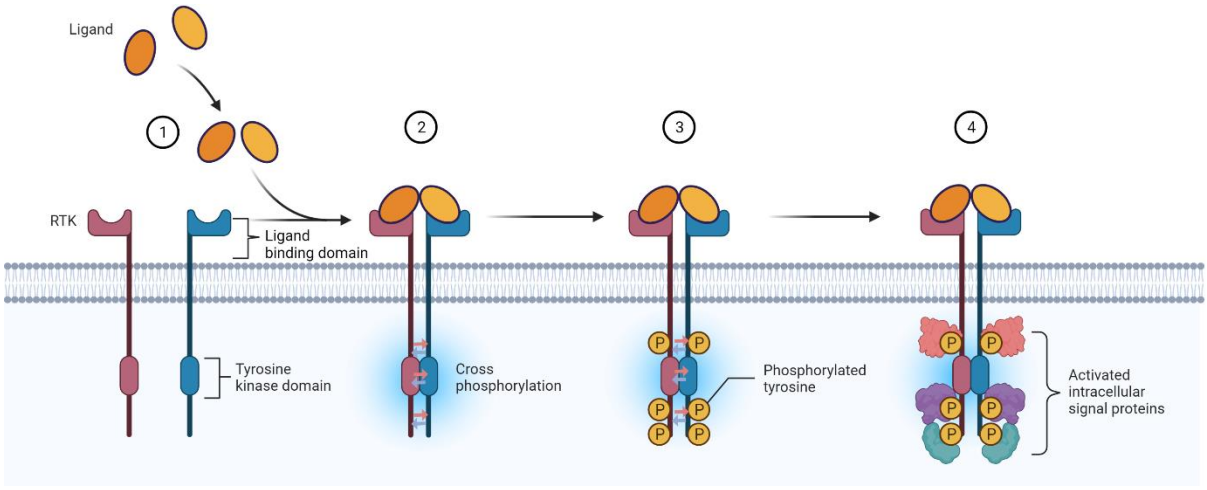


Figure 3 Overview of RTK activation. RTK (receptor tyrosine kinase); P (tyrosine phosphorylation)**.**

RTK signaling dysregulation has been linked to many human diseases, especially cancer (*88, 89*). Several reasons lead to pathological RTK signaling for example receptor overexpression or mutations within receptors. Examples of such instances include: the endothelial growth factor receptor (EGFR) contributes to the progression of colorectal cancer, pancreatic cancer, bladder cancer, breast cancer and liver cancer (*90-94*); Fibroblast growth factor receptor (FGFR) is altered in breast cancer, bladder, prostate and lung cancers (*95, 96*); Vascular endothelial growth factor receptor (VEGFR) is dysfunctional in colorectal, breast cancer, non-small-cell lung and renal cancers (*97-99*); Platelet-derived growth factor receptor (PDGFR) is

found to be altered in colorectal, melanoma, lung, glioblastoma, bladder and prostate cancers (*100-102*); Anaplastic lymphoma kinase (ALK) alteration was found in lung and breast cancers (*103, 104*); Insulin-like growth factor receptor (IGF1R) alteration were found in melanoma, Ewing's sarcoma, breast and colorectal cancers(*105-107*).

Most evidence shows that RTKs act as oncoproteins that assume responsibility for malignant cell transformation, proliferation and survival. Due to their oncogenic capacity, RTKs became attractive targets in cancer drug discovery. Many monoclonal antibodies and small molecular inhibitors targeting RTKs were designed but only showed a good therapeutic effect in a small number of patients.

1.4 THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR SYSTEM

Among all RTKs, IGF1R is probably the most well-explored targets for cancer treatment (*108, 109*). The IGF1R system constitutes a highly complex signaling machinery that consists of receptors, ligands, and ligand-binding proteins. At the cell membrane level, there are five transmembrane receptors: Insulin receptor (IR), Insulin-like growth factor receptor (IGF1R), IR/IGF1R hybrid receptor (half IGF1R and half Insulin receptor) (*110, 111*), Insulin-like growth factor 2 receptor (IGF-2R), and Insulin-related receptor (IRR) (*108, 112*). At the extracellular level, three canonical ligands are involved in the activation of the IGF1R system: Insulin, IGF-1, and IGF-2. All of them have critical roles in cell growth, proliferation and several other essential processes (*109*). Most IGFs in circulation are inactivated through binding to IGF-binding proteins. It has been demonstrated that, 90% of the IGF in circulation is regulated by IGF-binding proteins (IGFBPs), mainly IGFBP3 (*113*). Hence, IGFBP1-6 are important elements in regulating the activity of the IGF1R system.

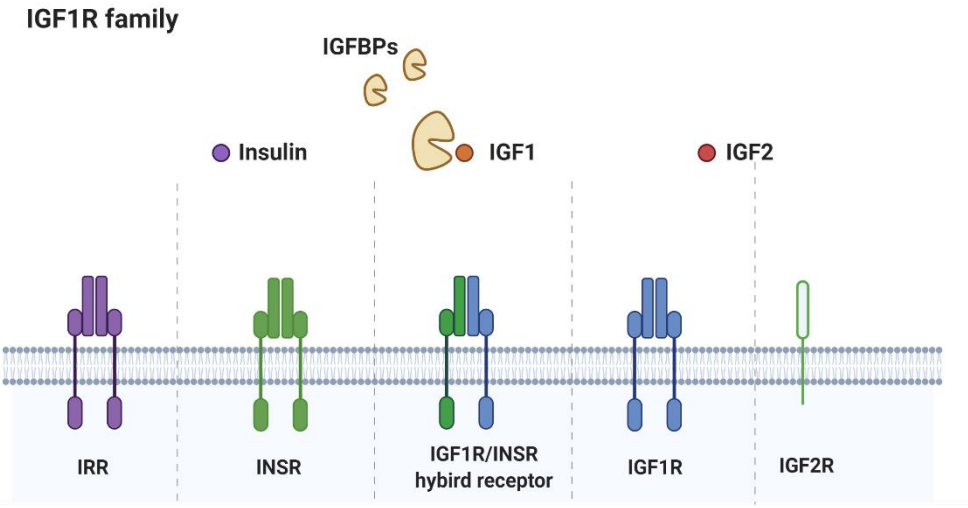


Figure 4 Overview of IGF1R family.

1.4.1 IGF1R

The IGF1R is widely expressed throughout different tissues and regulates cell growth, proliferation, and differentiation. The relationship between IGF1R structure and its function has been widely studied, mutational studies have indicated the residues essential for effectors binding and downstream bioactivities. IGF1R, unlike other RTKs, is expressed as a dimeric receptor on the cell membrane surface. It is comprised of two α-subunits and two β-subunits linked by disulfide bonds: the α-subunits contain a cysteine-rich domain for ligand-binding while β-subunits consist of an extracellular, a short transmembrane domain, and intracellular parts. Three domains are defined within the β-subunits: a juxtamembrane domain, a tyrosine kinase domain, and a c-terminal tail (*114, 115*). The juxtamembrane domain contains an NPXY motif (residues 947-950) (*116*). Once phosphorylated, the juxtamembrane domain Y950 acts as docking sites for signaling substrates, like IRS1 and Shc, then the receptor recruits other signal modulators to activate downstream signaling. This domain is also crucial for receptor internalization that ends signaling (*117*); The tyrosine kinase domain has ATP binding sites at positions 976-978, and also an activation loop (A-loop) which is critical for IGF1R autophosphorylation (*118*); The c-terminal tail of the IGF1R (residues 1229-1337) contains several regulatory elements essential for IGF1R function. It is like a control panel for IGF1R trafficking, which is related to multiple biological functions (*119*).

1.4.2 IGF1R activation and signaling

1.4.2.1 IGF1R canonical signaling

Canonical IGF1R signaling occurs via kinase activation. This process can be divided into several steps: (1) ligand-binding leads to conformational changes and phosphorylation of the A-loop tyrosine (Y1131, Y1135, Y1136) by their dimer partner. (2) Once A-loop is phosphorylated, this increases the kinase power of the IGF1R and, in turn, phosphorylates multiple tyrosine residues at the juxtamembrane domain (Y943, Y950) and c-terminal (Y1250, Y1251, and Y1346), creating docking sites for signal transducers. (3) Intercellular signal transduction molecules such as Shc and IRS can be recruited to docking sites (Y950, for instance), then trigger the downstream signaling activation (*120-122*) (Figure 4).

After Shc and IRS binding to the receptors juxtamembrane domain, both MAPK/ERK pathway and PI3K/AKT pathways can be activated. Grb2 recognizes the phosphorylated IRS and Shc via the Src homology 2 (SH2) domain. Then SOS, a Ras exchange factor, forms a complex with Grb2 and links IGF1R to induce RAS activation (*123*). It is believed that the Ras-GTP (activated RAS) interacts with Rafs to phosphorylate and activate Raf/MAPK/MEK/ERK axis (*124*). The activated ERKs translocate to the nucleus and phosphorylate transcriptional factors in IGF1 induced bioactivities (*125-128*).

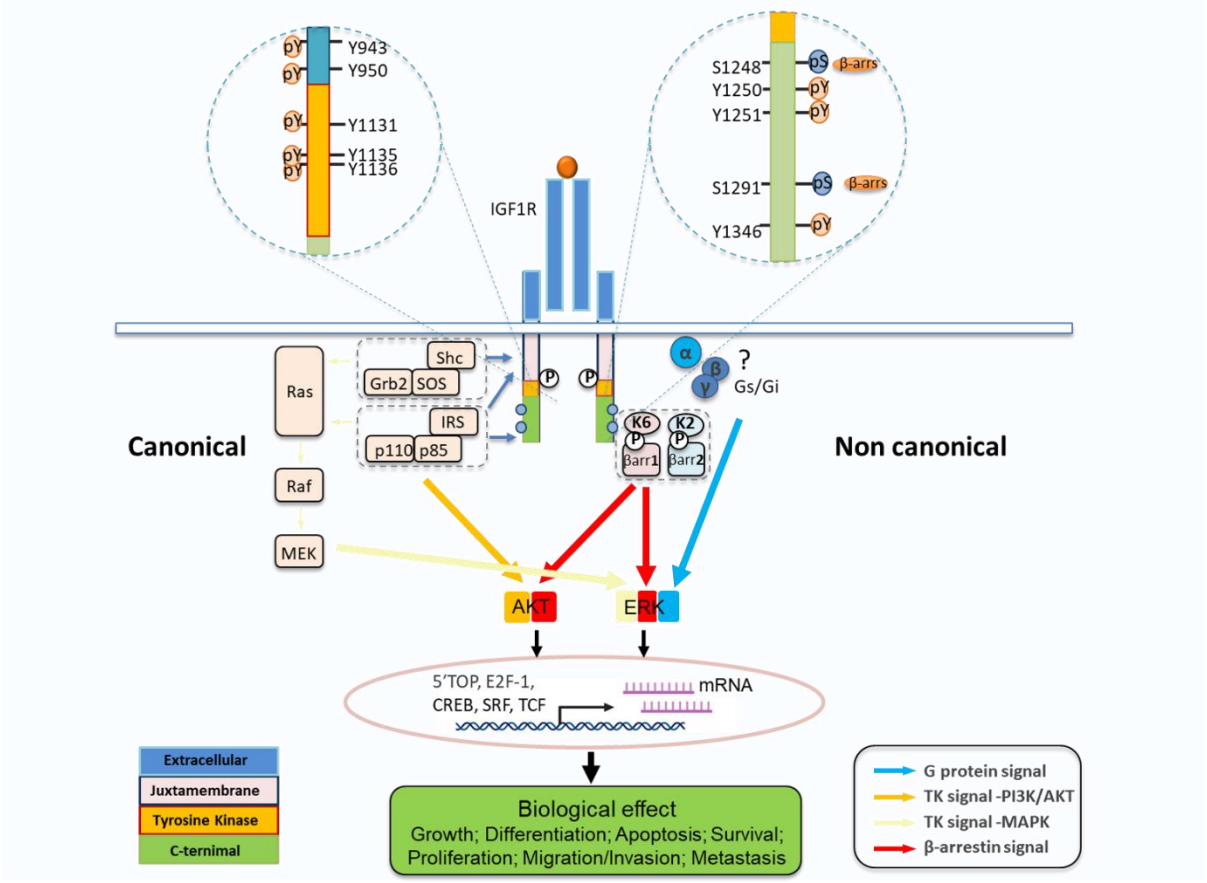


Figure 5 IGF1R canonical and non-canonical signaling.
Canonical signaling (kinase dependent signal): IGF1 binding induces IGF1R autophosphorylation, then receptor translate it into two main signal cascades activation: MAPK-ERK and PI3K-AKT; Non-canonical signaling (kinase independent signal): IGF1R can also utilize G protein and GRK/β-arrestin system to induce signaling downstream of IGF1R. Both canonical and non-canonical signals translocate to the nucleus and phosphorylate transcriptional factors, resulting in biological effects. Extracellular domain (Blue), Juxtamembrane domain (Pink), Tyrosine kinase domine (Orange), C-terminal (Green). pY (phospho-tyrosine), pS (phospho-serine), βarr (β-arrestin), α (Gα subunits), βγ (Gβγ subunits)

For the PI3K-AKT-mTOR cascade, the regulator subunit p85 interacts with IRS and activates p110 catalytic subunit. PI3K activation generates phosphatidylinositol-3,4,5-trisphosphate (PI3,4,5, P3) through phosphatidylinositol-3,4-bisphosphate (PIP2) phosphorylation at the intracellular side of the plasma membrane (*129*, *130*). AKT binds to PIP3 at the plasma membrane via their PH domain, promoting PDK1 to phosphorylate AKT at the catalytic

phosphorylation site (Thr308), resulting in activation of AKT and its related substrates such as Bad, mTOR, FOXO, Bcl-2, GSK-3β (*131-133*). These signaling activations control several biological effects such as cell proliferation, survival (*134*), transformation (*135, 136*), migration and anti-apoptosis (*137, 138*).

1.4.2.2 IGF1R non-canonical signaling

Kinase activation following IGFs binding is not the only way to transduce IGF1R signaling. It has been demonstrated that IGF1R signaling can also be mediated by non-canonical ligands (e.g. LL_37) (*139*), by system biased of the GPCR components (GRK/β-arrestin system) (*140-142*)and by biased receptors (e.g. truncation of the C-terminus) (*143*). By utilizing GPCR signal elements, IGF1R can activate kinase-independent signaling, termed non-canonical signaling.

IGF1R and G proteins

There is sufficient evidence indicating that signaling transduction by RTKs and GPCRs do not operate in an isolated manner. GPCRs and RTKs can transactivate each other, or RTKs can also directly engage GPCRs downstream components for their signaling transduction (*144, 145*). For instance, it has been demonstrated that IGF1R can use the ultimate signal component of GPCRs, the G protein. In mouse fibroblasts and rat neuronal cells, the MAPK pathway via IGF1R was sensitive to pertussis toxin, an inhibitor that blocks the GDP/GTP exchange from Gαi (*146, 147*). This study proved that G protein signaling is involved in IGF1R function. Following this promising hypothesis, other researchers determined that IGF1R specifically use Gi2 to mediate adenylyl cyclase activity, resulting in decreasing cAMP levels in the cytoplasm (*148*). Moreover, the direct physical interaction between IGF1R and Gαi/Gβγ has been proved by using immunoprecipitation (*149*). Altogether, these studies have shown that the IGF-IR can employ different G proteins for their downstream signaling modulation.

IGF1R and GRK/β-arrestin system

In addition to G proteins, the GRK/β-arrestin system was also found to be used by IGF1R. It has been shown that the GRK/β-arrestin system mediates IGF1R signaling, trafficking, and corresponding biological effects. The starting point of this serial study was the identification of Mdm2 as a ubiquitination E3 ligase for the IGF1R (*150*). During the ubiquitination process, β-arrestin 1 serves as a scaffold for Mdm2, linking it with IGF1R. Both β-arrestin 1 and Mdm2 can be co-immunoprecipitated with the IGF1R (*151*). These findings opened the avenues to exploring the functional roles of β-arrestins in IGF1R desensitization.

As the master regulators of GPCRs, β-arrestin isoforms have different functional roles in controlling IGF1R signaling: β-arrestin 1 tends to bind to ligand-occupied IGF1R, resulting in receptor degradation. β-arrestin1 can induce kinase-independent ERK activation, which is cancer-protective (*147, 152*); β-arrestin 2 favors binding to the ligand-unoccupied receptor. β-arrestin 2 competes with β-arrestin1 on IGF1R trafficking. β-arrestin 2 protects the IGF1R from IGF1 induced receptor degradation and inhibits the β-arrestin 1 signaling (*141*). However, the biological roles of β-arrestin 2 signaling need to be further explored. Another functional element of GPCRs, GRKs, has also been demonstrated as modulators of IGF1R signaling. For example, GRK5/6 depletion impaired the signaling downstream of IGF1R, whereas GRK2 knockdown increased IGF1R signaling activation (*152*). GRK 2 and GRK6 phosphorylates the serine residues at the c-terminal of IGF1R, creating binding sites for β-arrestins. The particular serine phosphorylation site affects the IGF1R/β-arrestin interaction: GRK2 mainly phosphorylates serine 1248 to promote transient β-arrestin receptor binding, resulting in a short-wave signal, while GRK6 phosphorylates the serine residue 1291 to induce β-arrestin stable receptor binding, and a prolonged-wave signal (*152*).

1.4.3 IGF1R trafficking

Ligand binding induce receptor autophosphorylation, and once the receptor is phosphorylated, this triggers an endocytosis mechanism via recruitment of adapter proteins. Subsequently, the receptors will soon be internalized within an endosome, where then it can be either degraded (via lysosomal/proteasomal system) or recycled back to the cell membrane in an inactivated state (Figure 5). The GRK/β-arrestin system is critical in this sorting process. Different GRK/β-arrestin combinations may support distinct functions in receptor trafficking: GRK2/β-arrestin 2 is more likely to lead receptors into recycling, while GRK6/β-arrestin 1 controls receptor degradation (*153*).

The result of an internalized IGF1R is highly dependent on ubiquitination. Similar to other protein ubiquitination, E1 (activating), E2 (transferring), E3 (legating) enzymes are required in IGF1R ubiquitination. The IGF1R can be either polyubiquitinated or monoubiquitinated, however the functional roles of different ubiquitination on IGF1R trafficking are not clear. There are at least four E3-ligases that have been identified to orchestrate IGF1R expression and function: MDM2 (*150*) , NEDD4 (*154*) , C-CBL (*155*) , HRD1 (*156*). Some of them require specific adaptor proteins (for instance, Mdm2 is recruited to the IGF1R via β-arrestin 1). The interaction between IGF1R and various E3-ligases has been extensively described (*153, 157*).

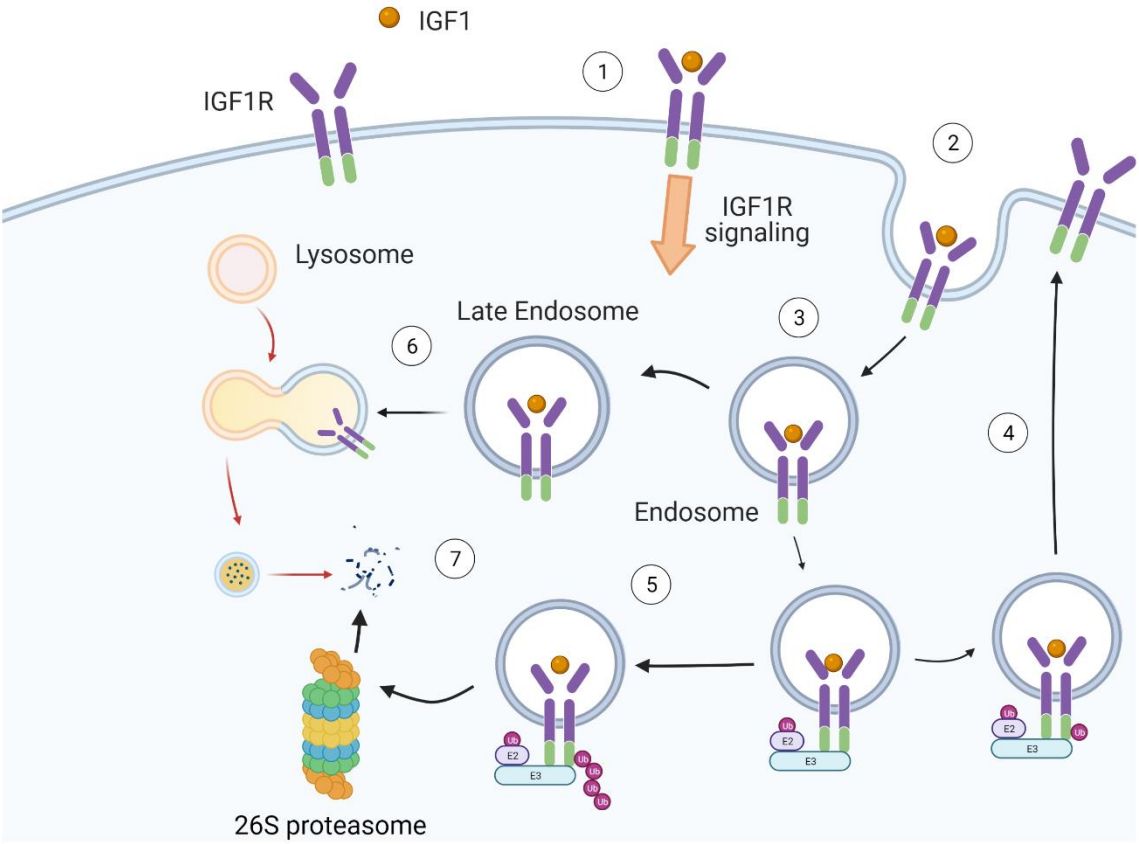


Figure 6. The overview of IGF1R trafficking (internalization and degradation).

1.4.4 IGF1R and cancer therapy

Extensive experimental studies across several cancer types have shown a crucial role of IGF1R in the development of tumorigenesis by regulating cell proliferation, differentiation, transformation, survival, and apoptosis (*158-160*). In *in vivo* models, overexpression of IGF1R induced malignant transformation of mouse fibroblasts, while, on the other side, IGF1R inhibition (expression or signaling) suppressed the tumor development and growth (*161-165*). More than that, the anchorage-independent growth which allows transformed cells to survive the process of cancer metastasis, was facilitated by IGF1R mediates signaling (*166*). IGF1R is frequently overexpressed and/or activated in various cancers, including colon (*167, 168*), breast (*169-171*), ovarian, hematopoietic, rhabdomyosarcoma, renal, prostate (*172, 173*), and lung cancer (*174*).

All the above studies recognize the IGF1R system as an attractive target for cancer treatment. Thus, developing an anti-IGF1R drug-based regimen has become a major cancer research area for the past three decades (*175, 176*). Based on the structure of IGF1R, extensive IGF1R targeting monoclonal antibodies and tyrosine kinase inhibitors (TKIs) have been developed and progression into clinical trials. Not unexpectedly, preclinical tests obtained encouraging results, demonstrating general benefits of inhibiting IGF1R in cancer. Researchers and

pharmaceutical companies held high hopes for the clinical application of anti-IGF1R therapy. However, clinical trials did not produce the desired outcomes, and only patients with selected types of cancer (non-small cell lung cancer, breast cancer, Ewing's sarcoma) gained benefits from it (*177*). There may be several reasons behind the unpromising outcomes of anti-IGF1R therapy in clinical trials, such as constitutively activation of downstream signaling; non-canonical signaling activation; absence of IRS1; crosstalk with other receptors (*177*). To sum up, enhancing patient response to IGF1R treatment is still a challenge, and the understanding of IGF1R should be updated.

1.4.5 **IGF1R and biased signaling**

The concept of biased signaling is not exclusive to the GPCR signaling paradigm, it is also fully relevant for IGF1R. Since recognition of the involvement of β-arrestin signaling (kinase-independent) at IGF1R, there are at least two distinct signaling arms downstream IGF1R: tyrosine kinase and β-arrestin. It has been shown that in certain conditions IGF1R may induce preferential β-arrestin signaling, indicating a "balanced" or "biased" behavior. IGF1 as a balanced ligand can induce both tyrosine kinase signaling and β-arrestin signaling, whereas β-arrestin biased agonists such as Figitumumab (CP-751,871), an anti-IGF1R antibody, binds to the receptor, resulting in kinase inhibition but β-arrestin biased signaling activation (*178*). These results demonstrated that similar to GPCRs, IGF1R can act in a biased manner, which helps improve our understanding of IGF1R.

Several treatments were proved to induce IGF1R signaling in a biased manner. PPP (picropodophyllin), a tyrosine inhibitor, induces IGF1R degradation and β-arrestin1 signaling activation (*143*). Furthermore, the study of LL37 identified this peptide as a natural IGF1R biased agonist (*139*). Thus, it is conceivable that controlling of β-arrestin signaling downstream of IGF1R, e.g. downregulating IGF1R without biased β-arrestin signaling, could be a potential therapeutic strategy in cancer treatment.

2.RESEARCH AIMS

The overall aim of this thesis is to explore the potential of IGF1R targeting though GPCR components in cancer treatment.

Study 1. To investigate the molecular mechanisms of the IGF1R/β-arrestin 1/p53 axis in sustaining the malignant phenotype of conjunctival melanoma.

Study 2. To investigate the contrasting abilities of GRK2 and GRK6 isoforms in controlling IGF1R trafficking as a potential target to achieve unbiased IGF1R downregulation in cancer cells

Study 3. To investigate the therapeutic potential of system biased downregulation of the IGF1R as a multi-hit approach in colon cancer.

Study 4. To explore the mechanism of how G protein signaling alters the IGF1R signaling/ trafficking and its therapeutic potential in cancer treatment.

3 MATERIALS AND METHODS

The details of materials and methods have been mentioned in individual papers. Here I only summarize some important materials and methods we used.

3.1 Cell culture

The cell lines we used in different studies are listed in the table below.

	ATCC	Other source
Paper 1		CM2005.1, CRMM1 and CRMM2 (Conjunctival melanoma cell lines) BE and DFB (Skin melanoma cell lines)
Paper2	HEK293T; A673, CADO, RDES, SKES, and SKNMC (Ewing sarcoma cell lines); U2OS and Saos-2(osteosarcoma cell lines)	Mouse embryonic fibroblasts (MEF) wild-type (WT) and knockout for β-arrestin1 (KO β1). MEFΔCT*
Paper 3	HT-29, HCT-116, SW48, SW480, LS123, LS174T, WIDR, colo320, DLD1 and LS1034 (Colon carcinoma cell lines)	
Paper 4	HEK293T; U2OS and Saos-2 (osteosarcoma cell lines)	MEF and MEF (P6, 46, 56, 96) ** HEKΔCT***

Table. The list of cell lines used in individual studies.

* MEF with an IGF1R–null background cells (R-) stably transfected with IGF1R with C-terminal tail truncation at residue 1245.

** MEF lacking an IGF1R (R-) cells, stably expressing either full-length IGF1R (P6), an IGF1R with mutation in substrate binding site (SBS) of IGF1R (46), an IGF1R lacking the C-terminal domain (56) or one with mutation in SBS and lacking C-terminal domain (96).

*** HEK-293T cells overexpressing SBP-tagged IGF1R with C-terminal tail truncation at residue 1251.

3.2. Western blot and densitometry analysis

Western blot (WB) is a method to detect proteins of interest. Protein samples were dissolved in lithium dodecyl sulphate (LDS) sample buffer and analysed by SDS-PAGE with 4-12% Bis-Tris gels. Upon separation, proteins were transferred to nitrocellulose membranes at

appropriate voltage. Membranes were then blocked in bovine serum albumin (BSA) and 0.1% Tween 20 in tris-buffer saline (TBS). Primary antibody in BSA was incubated overnight at 4 °C. Following 3 x 10 min washing (TBS-T), membranes were incubated with secondary antibody, either with fluorescent conjugated IRDye and detection with LI-COR Odyssey, or horseradish peroxidase conjugation and chemi-luminescence detection with ECL substrate and exposure to X-ray film. After getting the results from either detection machine, band intensity was measured by Image Studio or ImageJ and displayed relative to their respective loading control across multiple experiments. This method was used in all four papers to detect target proteins.

3.3 Immunohistochemistry (IHC)

IHC is another method to detect the protein of interest and its location in cells or tissue. Immunostaining was performed using the standard avidin–biotin complex (ABC) technique. Spheroids or cell pallets were fixed in 4% PFA and embedded in paraffin. After deparaffinization, rehydrated spheroid sections were pre-treated by microwaving for 10 min in 0.1 M citrate buffer at pH 6.0 and incubated with blocking serum (1% BSA) for 20 min followed by incubation with the primary antibody overnight at 4 °C. A biotinylated IgG was used as a secondary antibody, followed by the ABC complex. The peroxide reaction was developed using 3.3-diaminobenzidine tetrahydrochloride (0.6 mg/mL with 0.03% hydrogen peroxide) and processed for hematoxylin counterstaining. With the exception of paraffin embedding, the same fixation and staining protocol was applied to cell slides. Sections and cell slides were scanned and analyzed with Qu-path software. This method was used to detect IGF1R expression in mice tumor (Paper 2), p53 expression in BE, DFB, CM2005.1, CRMM1 and CRMM2 cells (Paper 1), ki67 in spheroid growth assay (Paper 1,3).

3.3 Biological effects in vitro

3.3.1. Cell viability assay

PrestoBlue cell viability assay was used for measuring cell viability according to the manufacturer's instructions (Invitrogen, CA, USA). Briefly, fluorescence was measured with excitation at 560 nm and emission at 590 nm using a Tecan Infinite 1000 plate reader. Cell numbers were interpolated from a standard curve of fluorescence measurement from known numbers of cells. This method was used in all four papers to check the cell viability.

3.3.2. Spheroid formation and growth assay

The spheroid 3D model is one of the critical methods used in Study 1 and Study 3. This method is a good model for drug testing. There are several advantages of this model: 1. Compared with the 2D model, tumor spheroids are closer to the process of tumor growth in the human body. 2. The experiment time is shorter (5-14 days), comparing with *in vivo* model. Therefore, the process is easy to handle. 3. We can use this model to verify several biological effects like tumor growth, cell apoptosis, cell migration, and invasion. And it is a good model to induce hypoxia condition. But nothing is perfect; there are still some disadvantages of this method: 1. Not all cell lines can form spheroids, even in Matrigel. 2. The signaling network may change a lot. 3D model experiment results sometimes may not in line with the 2D. 3. It is still not completely mimicking the tumor growth in the human body. 5×10^3 of cells were seeded in each well in a Nunclon Sphera 96-well U-bottomed plate in their normal growth medium (10% FBS and 1% P/S) and incubated for 3 days at 37 °C in a 5% CO2 incubator. EVOS Cell Imaging Systems was used to capture images of the spheroid (×4 objective). Quantification of the area of the spheroids was performing using Adobe Photoshop CS5.1.

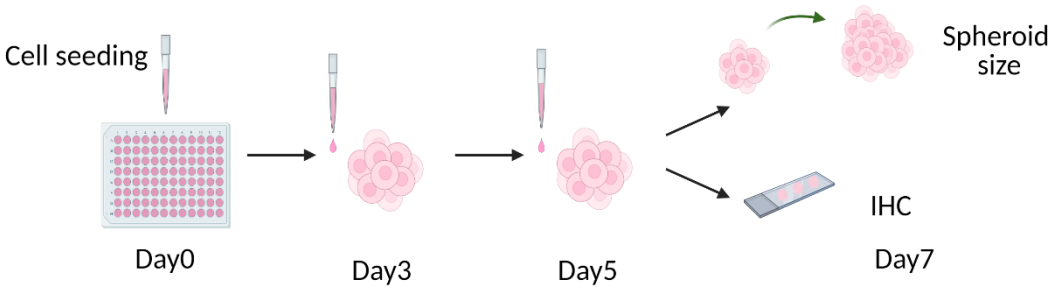


Figure 7. Workflow for tumor spheroid (3D model) formation experiments.

3.4 Biological effects in *in vivo*

3.4.1 Zebrafish

Zebrafish larvae are a simple and effective experimental model in biomedical research. It has several advantages including short generation time, easy breeding and low-cost (*179, 180*). We established xenograft model in zebrafish embryos to test tumor growth and metastatic potential. CM-Dil stained cells were resuspended in 1 mL PBS containing 2% polyvinylpyrrolidone to avoid clogging of the microinjection capillary. At 2 days post fertilization (dpf), embryos were injected with approximately 100 cells in the perivitelline space (PVS). The PVS was chosen as

an accurate model of both tumor growth and metastatic potential (*181*). One hour post injection, embryos were imaged using high-throughput fluorescent microscopy. Selected embryos were randomly sorted to different experimental groups and received the first dose of treatment. The second dose of treatment was delivered at 4 dpf followed by final imaging at 5 dpf. The zebrafish larvae model was used in Paper 1,3,4. All Zebrafish work was carried out in accordance with local regulations by the Karolinska Institute Zebrafish Core Facility.

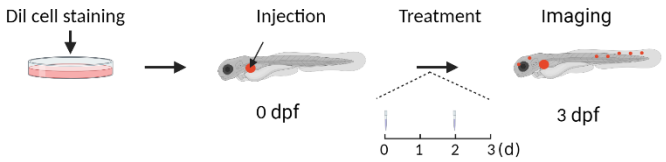


Figure 8. Workflow for zebrafish injection and treatment.

3.4.2 Mouse model

Mouse xenograft models have been established long time ago. As the mammal, it can mimic not all, but most conditions in humans (*182-184*). However, the duration of each experiment is much longer compared with zebrafish xenograft model. Cells were cultured to a confluence of 75%, harvested with trypsin/ EDTA, washed twice, and resuspended in PBS. Xenografts were inoculated in 5- to 8-week-old male nude mice by subcutaneous (s.c.) injection of $5*10^6$ cells in 0.1 mL sterile saline. Tumor volume was measured every 4 days. At tumor mean volume of 65 mm3, mice were randomly assigned to receive intraperitoneal (i.p.) injections. Mice were monitored for side effects and sacrificed at 32 days after commencement of treatment, when control-treated tumors reached 1,000 mm^3. Collected tumors were measured and split in two halves that were further processed either for histology (paraffin) or frozen for protein/RNA extraction. The mouse xenograft model was used in Paper 2. Xenograft studies were approved by the MD Anderson Cancer Center (MDACC) Institutional Animal Care and Use Committee, and all animal care was in accordance with institutional guidelines.

3.5 Statistical analysis

Where indicated, three independent experimental replicates containing normally distributed data (data underwent D'Agootino–Pearson normality testing) of two conditions were compared using a two-tailed unpaired t-test, and when more than two conditions, using an ANOVA, using

GraphPad Prism (version 8.2.1, GraphPad Software, CA, USA). Experimental design included a threshold value of $P = 0.05$ for testing any null hypothesis. The variances of the experimental groups that were being compared were not statistically different. Data expressed with error bars indicate mean ± SEM from three independent experiments. Significance is given as $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

4 RESULTS AND DISCUSSION

4.1 STUDY 1: IGF1R IS A MOLECULAR DETERMINANT FOR RESPONSE TO P53 REACTIVATION THERAPY IN CONJUNCTIVAL MELANOMA

Background and rationale:

Conjunctival melanoma (CM) is a type of mucosal melanoma, which whilst rare, the prevalence of CM has been increasing in recent years (*185*). Despite the more accurate diagnostic tools and methods, there has been no significant increase in CM patients' survival rate due to the lack of improvement in CM treatment (*186*). Thus, novel therapeutic approaches are urgently needed for CM treatment. A recent study shows that a UVR-related DNA-damage signature is associated with the pathogenesis of CM, which indicates some treatments used for skin melanoma (largely UVR-driven) could also be a therapeutic strategy in CM treatment (*187*). Our previous study showed that p53 reactivation, via the IGF1R/β-arrestin/MDM2 axis, was a promising strategy for skin melanoma treatment (*188*). In this study, we aimed to investigate the therapeutic potential of p53 reactivation in CM treatment.

Results and Discussion:

This study used three CM cell lines (CM2005.1, CRMM1, CRMM2). First, we tested the effect of Mdm2 inhibition on CM cell viability by using Prestoblue cell viability assay. We demonstrated that Mdm2 inhibition (either Mdm2 antagonist Nutlin-3 or Mdm2-specific siRNA) increases the p53 expression level and inhibits the cell viability of CM in a dose- and time- dependent manner. The cells were more sensitive to Nutlin3 than Mdm2 depletion by siRNA, which indicated a p53 independent mechanism behind Mdm2 in CM cell viability. Mdm2 has been described as an E3 ligase for IGF1R (*151*), we next investigated the effects of Nutlin3 on IGF1R expression and signaling. All three cell lines could respond to IGF1 stimulation and activate downstream signaling, which indicated that IGF1R may control CM growth. Nutlin3 degrades IGF1R and inhibits cell proliferation in a ligand-dependent manner.

It has been shown that β-arrestin1 works as an adaptor protein to orchestrate IGF1R/Mdm2 interaction (*189, 190*). Next, we tested the dependency of the Nutlin3-induced IGF1R degradation on β-arrestin1. We knocked down β-arrestin 1 by transfection of targeting siRNA. Western bolt analysis showed that β-arrestin1 depletion by siRNA not only protects the receptor from Nutlin3 induced receptor degradation but also augments the inhibition effect of Nutlin-3 on CM cell survival. Afterwards, we explored the functional role of β-arrestin 1 in this process; β-arrestin 1 overexpression enhances Nutlin-3 induced receptor degradation and

inhibits p53 expression. Altogether, these results indicate that β-arrestin 1 have an essential role in Nutlin-3 induced receptor degradation and p53 reactivation.

Mitomycin (MMC) is a classical adjuvant treatment for CM patients, and it induces p53 activation via genotoxic stress. So next, we compared these two alternative p53-targeting strategies on the cell biological activities of CM. We used both 2D and 3D *in vitro* models to test the inhibitory impact of MMC and Nutlin-3 on cell growth. MMC, Nutlin-3, and combination treatment reduced the tumor growth by about 30%. Even though the tumor is more sensitive to MMC treatment, the cell proliferation of CM can be totally inhibited by Nutlin-3, indicated from ki67 staining in the spheroid model. We verified this result in zebrafish CM xenograft models; similar to the result in 2D and 3D culture, Nutlin-3 treatment is most effective in impairing tumor growth and metastatic potential.

Conjunctival melanoma (CM) is the most frequently presenting mucosal melanoma and distinguishes itself within this category as being the single one with UVR exposure as an etiologic factor. There are currently no consensus guidelines on postoperative adjuvant therapies to decrease the risk of recurrence and metastasis. In search for a better understanding of the molecular mechanisms driving this particular melanoma, and to identify novel therapeutic targets for improved CM management, we specifically investigated the effects of destabilizing the Mdm2/p53 complex. We demonstrated that this Nutlin-3-induced IGF-1R degradation is controlled by a molecular switch represented by β-arrestin1, which manages both IGF-1R and p53 activity. We were able to validate in vivo relevance; in both cell models and zebrafish avatars, IGF-1R/p53 controlling via Nutlin-3 (Mdm2 inhibition) is more effective than the current golden standard for CM adjuvant therapy - mitomycin. Whilst illuminating the biased agonistic properties of Nutlin-3 for the β-arrestin pathway, our study provides fundamental insights into destabilizing p53/Mdm2/IGF-1R circuitry that could be developed for long-awaited therapeutic gain for CM patients. Furthermore, we developed a very important experimental model of spheroids and zebrafish xenografts to evaluate potential response of ocular tumors to various therapies.

4.2 STUDY 2: INHIBITION OF G PROTEIN–COUPLED RECEPTOR KINASE 2 PROMOTES UNBIASED DOWNREGULATION OF IGF1 RECEPTOR AND RESTRAINS MALIGNANT CELL GROWTH

Background and rationale:

Biased signaling described as the concept that GPCRs have the ability to activate one downstream pathway preferentially. This property is also found in IGF1R, a cancer relevant receptor tyrosine kinase (RTK). It has been shown that the effectiveness of anti-IGF1R therapy is restricted due to the activation of cancer protective biased β-arrestin (β-arr) signaling. Control of β-arr biased signaling targeting could therefore be a promising strategy to improve anti-IGF1R cancer therapy. It is known that the recruitment of GRKs is essential for activating β-arr signaling under IGF1R. In this study, we aimed to investigate the function of GRK2/GRK6 on IGF1R downregulation and their clinical application.

Results and Discussion:

To test how GRK2 and GRK6 modulate IGF1R expression and signaling, we transfected cells with GRK2 and GRK6 plasmids or targeted siRNA. The receptor expression and function was evaluated by western blot. The results demonstrated that GRK2 depletion and GRK6 overexpression enhances ligand-induced receptor degradation and sustained ERK activation (β-arr biased signal) in all cell lines. To further test the functional roles of GRK2(-) and GRK6(+) in malignancy potential, we assessed the anchorage and anchorage-independent cell growth. Our results showed that the cell viability was impaired by GRK2(-) and GRK6(+) in both anchorage-independent and adherent conditions. These results indicated that switching the balance to GRK6 could be a strategy to restrict malignant cell survival.

To check the pharmacological effects of GRK2 in Ewing's sarcoma (ES) cells, a GRK2 inhibitor, paroxetine (PX), was employed to test its effects on IGF1R expression and signaling. PX degraded IGF1R in a dose- and time- dependent manner without activating β-arrestin biased signaling. Additional experiments proved that PX induced IGF1R internalization, as well as degradation, dependent on β-arrestin 1. These results demonstrated that an IGF1R/β-arrestin 1 interaction was required in the PX-induced receptor downregulation.

To further confirm the effects of PX on the interaction between IGF1R and β-arrestins, we used coimmunoprecipitation(co-IP). IGF1R preferably bound to β-arrestin 2 at low ligand

conditions, while PX treatment switched the affinity of IGF1R from β-arrestin 2 to β-arrestin 1, which launched MDM2-dependent IGF1R ubiquitination and receptor degradation.

The following experiments categorized the PX effects on IGF1R expression and downstream signal in malignant ES cells. PX downregulated the IGF1R and lowered the receptor's response to IGF1. Previous studies showed that IGF1R downregulation via a β-arrestin 1 biased agonist was inefficient in ES treatment(*178*). We next compared the biological effects of biased (IGF1R targeting antibody, CP) and unbiased (PX) agonists. As measured by colony formation assay, PX decreased the countable colonies in a dose dependent manner while CP did not affect colony formation at any dose. In a mouse xenograft model, PX reduced tumor growth and decreased the IGF1R expression in the tumor tissue. Taken altogether, unbiased downregulation of IGF1R via GRK2 inhibition may be a good strategy for anti-IGF1R cancer treatment.

We show for the first time that system bias could be used for targeting IGF-1R. To our knowledge this represents a new paradigm for the entire class of RTKs and could be used as a starting point for the rational design of specific therapeutics targeting RTKs in any pathological conditions.

4.3 STUDY 3: G PROTEIN-COUPLED RECEPTOR KINASE 2/ BETA-ARRESTIN-1 SYSTEM BIAS INHIBITION OF IGF1R/EGFR DESTABILIZES THE METASTATIC PHENOTYPE OF COLORECTAL CARCINOMA

Background and rationale:

Colorectal carcinoma (CRC) is characterized by a high number of constitutive alterations of signal transduction pathways, generating complex, highly redundant growth-supportive networks (*191-193*). Experimental, epidemiological, and clinical data strongly demonstrate that CRC is highly dependent on a hyper-functional IGF1R system. However, all clinical trials targeting IGF1R failed in phase III. A possible explanation is the major paradox of IGF1R targeting; therapeutic strategies downregulating the receptors (e.g., IGF1R targeting antibodies) can activate cancer-protective β-arrestin-biased signaling (β-arr-BS). The challenge, of IGF1R downregulation without β-arr-BS activation, was recently solved within a "system bias" model through pharmacological inhibition of GRK2 by paroxetine (PX) (*194*).

Considering the unique hyperactive CRC signal network, the present study aimed to investigate the impact of 'IGF1R system bias therapy' within rational multi-hit strategies, in CRC.

Results and Discussion:

We first tested the effects of PX on cell viability in a panel of human CRC cell lines using Prestoblue assay. PX treatment caused a dose-and time-dependent decrease in cell viability. It has been demonstrated that PX can downregulate IGF1R in Ewing's sarcoma. We next questioned if PX could downregulate IGF1R in colon cancer cells. We also included other RTKs, EGFR (GRK2 dependent RTK) and InsR(GRK2 independent RTK) in this experiment. Our results indicated that PX specifically downregulated and restricted signal transduction of GRK2-dependent IGF1R and EGFR but enhanced the signaling capabilities of the GRK2-independent RTK, InsR.

Hyper-sensitization of InsR impairs PX-effects on IGF1-induced and EGF-induced signaling. Therefore, we evaluated additional targets within RTK downstream signaling pathways. Using an online database, we compiled appropriate candidates and verified the inhibition effects of these inhibitors with/without PX in a cell viability assay. Combination treatment showed more efficiency than single PX treatment. In the 3D model, we compared the PX and combination treatments on spheroid formation. Like the 2D model, combination treatment showed an additive effect on 3D tumor growth. Overall, combined-agent therapy resulted in a significant decrease in cell viability compared to single-agent therapy.

To investigate the effect of combined-agent therapy on tumor metastasis, we used a 3D spheroid IGF-1 enriched model to mimic liver. The results showed that PX induced cell-death in the spheroid core but had no effects on the overall growth. More importantly, in IGF-1 rich conditions modeling liver metastasis, combinational treatment was more effective in restricting spheroid growth and inducing core cell death. These results were validated in zebrafish xenografts, where PX in combination with inhibitors restricted the growth of metastatic tumors.

TCGA analysis showed that GRK2 expression levels were higher in cancer tissue than in normal tissue. Taking a closer look at the CRC patient population, revealed that colorectal cancer patients with lower GRK2 and IGF1R RNA expression had better overall survival. The survival data, in *in vitro* and *in vivo* PX sensitivity data strongly support anti-IGF1R/EGFR via GRK2 system bias as a novel targeting approach for CRC patients.

4.4 STUDY 4: G PROTEIN ACTIVATION BALANCES SIGNALING PATHWAYS DOWNSTREAM OF THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR: THERAPEUTIC IMPLICATIONS FOR CANCER

Background and rationale:

Owing to the presence of an intracellular tyrosine kinase domain, and in accordance with all other members of the RTK family, phosphorylation is considered to be the central process controlling IGF1R signaling. However, over the last decade, this view has been challenged by data demonstrating non-canonical IGF1R signaling controlled by the GRK/β-arrestin system, effectors traditionally associated with the GPCR-family. Although identified earliest, the role of the third functional GPCR component, G proteins, is not fully delineated. Herein we investigated the function of heterotrimeric Gαβγ proteins downstream of IGF1R, within an updated RTK/GPCR functional dualism paradigm.

Results and Discussion:

It has been demonstrated that IGF1R signaling is sensitive to G protein inhibitors. We first verified the effect of G-protein inhibition on IGF1R downstream signaling in HEK-293T and MEF cell lines. The results showed the G-protein inhibitor impaired pathways downstream of IGF1R in both cell lines. In addition, IGF1R can transduce the signals of other ligands from the IIGF family, such as IGF-2 and Insulin. The results demonstrated that G protein inhibition impaired the IGF2 mediated signaling but not Insulin.

To further study the mechanism of G protein inhibition on IGF1R signaling, we next explored IGF1R expression. Western bolt analysis showed that Gi inhibition constrained the receptor phosphorylation upon IGF1 stimulation in a dose- and time- dependent manner, indicating less IGF1R expression on the cell surface. The result was confirmed using flow cytometer analysis and a biotinylation assay. These results demonstrate that IGF1R internalization is dependent and promoted by G proteins, while degradation is not.

The GRK/β-arrestin system has an essential role in IGF1R trafficking. Next, we explored the dependency of GRK/β-arrestin in G protein inhibition induced IGF1R downregulation. Cells were transfected with GRK and β-arrestin targeted siRNA and treated with G protein inhibitor; The G protein inhibition effects could be prevented by transgenic inhibition of GRK2 or β-arrestin 2 or with IGF1R mutants unable to bind β-arrestins.

Finally, we assessed the biological outcomes of G protein inhibition in *in vitro* and *in vivo* conditions. We investigated the effects of G protein inhibition on IGF-1 induced cell proliferation in U2OS (functional Mdm2) and Saos2 (non-functional Mdm2). In *in vitro* and *in vivo* results indicated that G protein inhibition ultimately constrained IGF-1 driven proliferation and metastatic potential of malignant cells.

5 CONCLUSIONS

The main conclusion of each paper is summarized as follows:

***Study 1*:** This research reveals the possibility of controlling IGF1R and p53 as a therapeutic strategy, which reduces the risk of recurrence and metastasis, thus revealing CM targeted therapy. Whilst illuminating the biased agonistic properties of Nutlin-3 for the β-arrestin pathway, our study provides fundamental insights into system bias approach for destabilizing p53/Mdm2/IGF-1R circuitry.

***Study 2*:** This study provides a novel "system bias" strategy: targeting IGF1R though the GRK/β-arrestin system. These findings revealed the molecular mechanism and biological function of β-arrestin biased signaling downstream of IGF1R and its promising therapeutic application in anti–IGF1R cancer treatment.

***Study 3*:** This study defines a potent two-step strategy: the first step towards dependent signaling pathways (MAPK or PI3K signaling) causes an oncogene addiction shift towards IGF1R/EGFR (cell growth slowly, but cells survive) while the second step (PX, GRK2 inhibitor) effectively shuts down the network needed for survival. This study reveals a strategy for adding an extra layer for control of system bias approach.

***Study 4*:** This work provides further insights into the molecular mechanism defining the RTK-GPCR dualism paradigm of the IGF1R, demonstrating its relevance with biological outcomes, and exposing the importance of controlling all facets for cancer treatment. Most important, this study provides information regarding biological roles of G-protein signaling downstream IGF1R and its potential role for anti-cancer therapy.

6 POINTS OF PERSPECTIVE

The following questions are my perspectives of anti-IGF1R strategy via the biased signal system for the future. Let us answer these questions in the next ten years.

1. What is the biological effect of the G protein signal downstream of IGF1R? Is it cancer protective or not? Is it possible to initiate G-protein biased signaling downstream IGF1R?

2. Will combination treatment with IGF1R biased agonists be a good way to improve the efficiency of multimodal (chemo-, radio, immune, targeted) therapy?

3. Anti-IGF1R via the biased signal system appears to be a good therapeutic rationale approach. Is this paradigm valid for other RTKs?

4. Does the biased β-arrestin signal or G signal downstream of RTKs have different functional roles in different types of cancer? Or in other diseases?

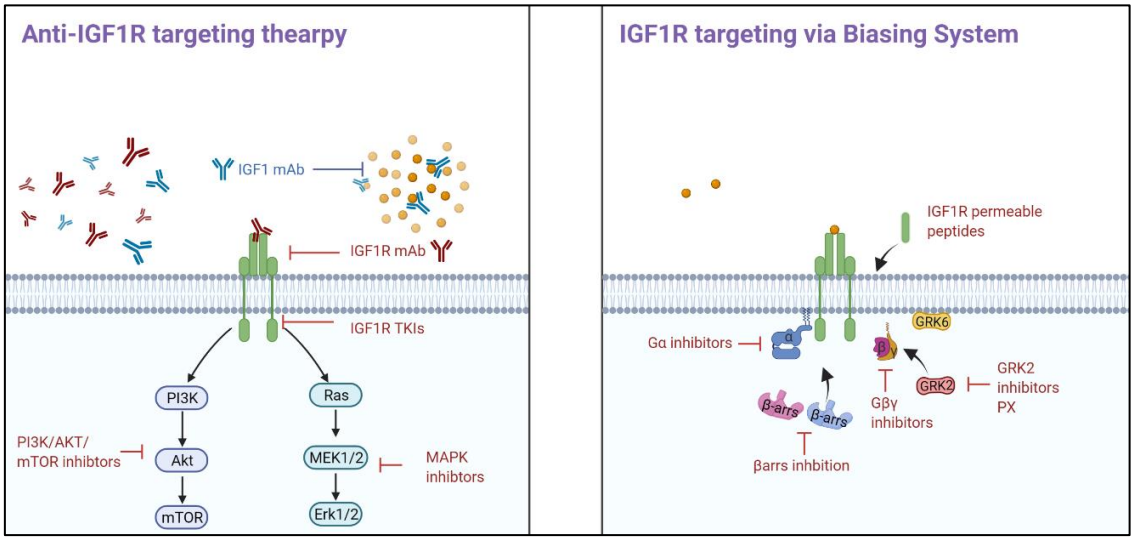


Figure 9 The approaches of anti-IGF1R targeting therapy and theri future implication.
Anti-IGF1R targeting therapy (left)**:** IGF1R can be targeted though several approaches: monoclonal antibodies (targeting IGF1R or IGF1), tyrosine kinase inhibitors, inhibitors of downstream signal components (MAPK, PI3K-AKT); Anti-IGF1R via system bias (right): More approaches should be tested in the future, for example inhibitors of GPCR components and IGF1R peptides.

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