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Signal Transduction Mechanisms Mediating the Regulation of Vascular G Protein-Coupled Receptors

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Abstract

Blood pressure homeostasis is controlled via a complex network of cell signaling mechanisms. Among the broad network of receptors and signaling molecules regulating blood vessel reactivity, members of the G protein-coupled receptor (GPCR) family are known to play a central role. GPCR activity represents a delicate, but coordinated balance between molecular mechanisms governing receptor signaling, desensitization, and re-sensitization. GPCR kinase 2 (GRK2) modulates multiple cellular responses through GPCR desensitization and alterations in GRK2 activity are considered to play an important role in the development of hypertension. The main premise of our study was to test whether the inhibition of GRK2 expression leads to alterations in vascular reactivity, vascular tone, and vascular smooth muscle cell (VSMC) signaling. Genetic knockdown of GRK2 expression results in a mouse that shows indications of intrauterine growth retardation phenotype and becomes spontaneously hypertensive at 8-12 weeks of age due to alterations in the balance between mechanisms regulating vasodilatation and vasoconstriction. The extensive loss of GRK2 expression favors an increased in vasoconstriction associated with an increase in peripheral resistance and this is likely due to the reduced $G\alpha_{q/11}$-coupled receptor desensitization. The vasodilatation in response to $G\alpha_S$-coupled receptor stimulation was also enhanced, but the increases in vasoconstrictor mechanisms dominate the physiological phenotype. In addition, VSMCs cultured from shGRK2-knockdown mice demonstrate an altered ERK1/2 and Akt/PKB signaling with age, as well as age-dependent increases in cellular proliferation and migration responses linked to $G\alpha_{q/11}$-coupled GPCR activation. Our results indicate that, as blood pressure increases in the shGRK2 mice, the expression of renal renin angiotensin system (RAS) components increases correspondingly and this has a strong impact on the regulation of both peripheral vascular resistance and sodium balance. The chronic activation of RAS also potentiates renal injury by inducing alteration in glomerular filtration rates and progression of renal fibrosis. Thereby, these intricate effects complement each other in the onset of hypertension. Finally, to enable definition of the role of Rab4GTPase on GPCRs re-sensitization, we have developed a vascular specific inhibitory Rab2S22N transgenic mouse and the documentation of the hypertensive phenotype is the first evidence for the existence of a causal relationship

between alteration in Rab4 activity and vascular GPCR signaling. Taken together, our findings indicate that the balance between mechanisms regulating vascular tone is significantly modulated by intracellular regulatory proteins underlying GPCR signal transduction.

Keywords: G protein-coupled receptors, heterotrimeric G proteins, G protein-coupled receptor kinases, desensitization, Rab4GTPase, re-sensitization, signal transduction, vascular smooth muscle cells, vascular tone, renin angiotensin system, hypertension

To the memory of my mother

Co-Authorship Statement

The data presented in Chapter 2 were previously published in *Journal of Biological Chemistry*, Feb. 2015, **290**(8): 5141-55. Dr. Hubert Van Tol was responsible for generation of the shGRK2 knockdown transgenic mice. Dr. Robert Gros performed the experiments presented in Fig. 2.1, 2.2, 2.3 and 2.4 with the exception of Fig. 2.1A, 2.1B and 2.4A that were performed by Elena Tutunea-Fatan. All other experiments presented in Fig. 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, and 2.12 were performed by Elena Tutunea-Fatan.

The data presented in Chapter 3 have been prepared for publication. All the experiments presented here were performed by Elena Tutunea-Fatan, with the exception of Tab. 3.5 that was generated by Dr. Robert Gros.

Elena Tutunea-Fatan performed all experiments presented in Chapter 4. Elena Tutunea-Fatan was responsible for generation of SM22αRab4N22S transgenic mice with microinjection assistance received from the Transgenic Core Facility of the University of Michigan.

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During the time spent on this research, I shared many thoughts, ideas, impressions or tasks with my laboratory colleagues. Particularly, I wish to express my appreciation and thank to Dr. Fabiana Crowley, Dr. Cornelia Walther, and Dr. Christina Vanderboor for their collaboration and friendship.

Last, but definitely not least, I need to extend my heartfelt thanks to my mother. Without her love and sacrifice, I would not have gotten this far in my life. Additionally, I would like to thank you to my husband, Remus, for his endless support and understanding.

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List of Abbreviations

Abbreviation	Full Name
α_1AR	Alpha 1 Adrenoreceptor
ACE	Angiotensin Converting Enzyme
ACTB	Actin Beta
ANG	Angiotensinogen
Ang II	Angiotensin II
AngII(SII)	Biased Angiotensin Receptor Agonist
ANOVA	Analysis of Variance
ARB	Angiotensin Receptor Blockers
ASMCs	Aortic Smooth Muscle Cells
AT_1R	Angiotensin Type 1 Receptor
AUC	Area Under the Curve
β_2AR	Beta 2 Adrenergic Receptor
BP	Blood Pressure
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin

Ca^{2+}	Calcium
CaCL2	Calcium Chloride
CAM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
D_1R	Dopamine Type 1 Receptor
DAG	Diacylglycerol
E15.5	Embryonic Day 15.5
ECs	Endothelial Cells
EGF	Epidermal Growth Factor
EGFP	Enhance Green Fluorescence Protein
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERK1/2	Extracellular Regulated Kinase 1/2
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate

G Protein (αβγ)	Guanine Nucleotide Binding Proteins
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDI	Guanine Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEP	Guanine Nucleotide Exchange Factor
GFR	Glomerular Filtration Rate
GPCRs	G Protein-Coupled Receptors
GRK2	G Protein Coupled Receptor Kinase 2
GRK4	G Protein Coupled Receptor Kinase 4
GRKs	G Protein Coupled Receptor Kinases
GTP	Guanosine Triphosphate
H & E	Hematoxylin & Eosin
HEK293	Human Embryonic Kidney 293
Hh	Hedgehog Signaling
HR	Heart Rate
5HT-1bR	Serotonin Type 1b Receptor
5HT-2bR	Serotonin Type 2b Receptor

ISO	Isoproterenol
IUGR	Intrauterine Growth Restriction
JAK	Janus Kinase
KCL	Potassium Chloride
LDL	Low Density Lipoprotein
LY294002	PI3K Inhibitor
MAPK	Mitogen Activated Protein Kinase
MLCP	Myosin Light Chain Phosphatase
M-PER	Mammalian Protein Extraction Reagent
mRNA	Messenger Ribonucleic Acid
NF-kB	Nuclear Factor kB
PDGF	Platelet Derived Growth Factor
PDK1	Pyruvate Dehydrogenase Kinase 1
PE	Phenylephrine
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol 4,5-Biphosphate

PKA	Protein Kinase A
PKB/AKT	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
p-RB	Phospho-Retinoblastoma
PSS	Physiological Salt Solution
PTC	Proximal Tubular Cells
RAS	Renin Angiotensin System
RGS	Regulated of G Protein Signaling
ROS	Reactive Oxygen Species
RT-PCR	Real Time-Polymerase Chain Reaction
SFM	Serum Free Medium
SM22α	Smooth Muscle 22 Alpha
SNS	Sympathetic Nervous System
STAT	Signal Transducer and Activator of Transcription
TBST	Tris-Buffer Saline with Tween-20
$TGF\beta_1$	Transforming Growth Factor Beta 1

TGN	Trans-Golgi Network
U0126	ERK Inhibitor
VSMCs	Vascular Smooth Muscle Cells

CHAPTER 1:

INTRODUCTION

1.1 G Protein-Coupled Receptor Family

The seven transmembrane-spanning domain or G protein-coupled receptors (GPCRs) represent the largest and the most diversified protein family of cell surface receptors (Lander *et al.*, 2001; Latek *et al.*, 2012). Current estimations assert that 1000 up to 2000 distinct receptors might exist if alternate splicing variants are also accounted for. This broad conformational variety allows GPCRs to be involved in a multitude of physiological processes including senses, behavioral and mood regulation, hemodynamics and intermediary metabolism control, as well as cellular growth, differentiation and apoptosis. Because of this diversity of stimuli to which GPCR respond to, it is not surprising that over half of all drugs used in the present clinical practice target GPCRs directly or indirectly (Flower, 1999).

The common trait of all known GPCRs is that they share a common central core domain comprised of seven transmembrane helices joined by three intracellular and three extracellular loops. The two additional cysteine residues that are present in most GPCRs are assumed to play an important role in packing as well as in the stabilization of certain conformations of the GPCRs. The extracellular region – characterized by a high structural diversity but a small movement during activation – plays a significant role in ligand binding Unlike the relatively stable shape of the extracellular zones, intracellular regions are known to be characterized by broad conformational modifications following receptor activation (Katritch *et al.*, 2012). Due to their localization within the inner cellular structure, intracellular regions interact extensively with a multitude of G proteins, arrestins as well as many other downstream effectors (Lefkowitz, 2000; Palczewski *et al.*, 2000; Palczewski, 2006; Cherezov *et al.*, 2007; Jaakola *et al.*, 2008; Rasmussen *et al.*, 2011b).

When it comes to the taxonomy of the GPCRs, several classification systems have been proposed. While an unitary classification scheme would have been advantageous from a variety of perspectives, the challenge to be overcome is related to the broad discrepancies in sequences that exist between invertebrate and mammalian GPCRs (Broeck, 2001). Some classifications are grouping the ligands based on how they are binding, while others rely on both physiological and structural characteristics. One of the common classifications – applicable to both vertebrates and invertebrates – is based on classes (involving letters from A to F) and subclasses (involving roman numbers) (Attwood and Findlay, 1994; Kolakowski, 1994; Bockaert and Pin, 1999; Josefsson, 1999; Graul and Sadee, 2001; Joost and Methner, 2002; Fredriksson *et al.*, 2003).

According to the widespread GRAFS taxonomy (Schioth and Fredriksson, 2005), the GPCR superfamily is split into five families, namely: glutamate (former class C), rhodopsin (former class A), adhesion (part of former class B), frizzled/taste2 (former class F), and secretin (part of former class B). Former classes D (fungal mating pheromone receptors) and E (cyclic AMP receptors) are not part of the GRAFS classification system, and that is essentially because they do not include human receptors.

Interestingly, not all receptors captured by the GRAFS classification are believed to signal through heterotrimeric G proteins, such that – at least when looking from this particular angle – the conventional “G protein-coupled receptor” term could be perceived as a somewhat of a misnomer, since in reality completely different signaling mechanisms could be activated. One of the well-described examples in this category is represented by the Frizzled receptors.

1.2 G Protein-Coupled Receptor Signaling

1.2.1 Receptor Structure-Function Relationship

The transmembrane localization and disposition of GPCRs – exposed to both extra- and intracellular stimuli – enable them to become a critical component for the transduction of the incoming extracellular messages into downstream intracellular responses. The presence of multiple enzymatic steps along the signaling pathway creates the premises for strong signal amplifications that occur even at low (5%) receptor occupancy rates (Arshavsky *et al.*, 2002).

Early studies proposed that the binding between agonist and transmembrane/extracellular domains of GPCR is accompanied by conformational changes of the receptor that are transmitted to its intracellular domains in contact with the G protein complex (Gether and Kobilka, 1998; Ridge *et al.*, 2003). These conformational changes can take either the form of a high-affinity "ternary complex" among the agonist-receptor-G protein or that of a low-affinity state (when G protein is absent) (De Lean *et al.*, 1980). However, more recent works have further enhanced this model in order to account for constitutively active GPCRs as well as full, partial, neutral, antagonist, and inverse agonists ("extended ternary complex model") (Samama *et al.*, 1993). The primary difference between these types of agonists resides in the preferred equilibrium state of the GPCR that can vary between active (R*) (full agonist binding to R* conformation), inactive (R) (inverse agonist binding to R), or unselective (neutral antagonist binding to either R* or R) (Lefkowitz *et al.*, 1993).

To account for all aspects of GPCR function, more comprehensive models (Kenakin, 2002; Kenakin, 2003) advocate for the existence of an "alternative ternary complex" that oscillates between a conformation required for G protein activation and a second one needed for receptor internalization. This model, originally proposed for β_2-adrenergic receptor activation (Swaminath *et al.*, 2004) has been later endorsed by the discovery of β-arrestin-dependent angiotensin AT_{1a} receptor internalization and signaling that continued to be present even when G protein coupling was lacking (Wei *et al.*, 2003).

1.2.2 G Protein-Dependent Signal Transduction

G proteins act as molecular switches for the intracellular signaling cascades and provide the link between the external stimulus and the intracellular effector enzymes. Heterotrimeric G proteins are a group of GTPases that share a common multi-subunit structure comprised of a guanosine triphosphate GTP-binding Gα subunit and a linked heterodimeric Gβγ subunit (Neer, 1995). Based on sequence homology, the Gα subunits were grouped into four main families, namely $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$. Alongside with Gα, five Gβ and twelve Gγ subunits were also identified.

The innate GTPase activity of the Gα subunit determines the hydrolysis of GTP to GDP and in turn, this will return the Gα to an inactive state that is characterized by a high Gβγ affinity. Because the re-association of Gβγ with Gα-GDP will cancel all effector interactions, the lifetime of Gα is believed to have a direct control over the signaling time of both Gα-GTP and free Gβγ (Siderovski and Willard, 2005). Since the activated GPCR catalyzes the exchange of GDP for GTP that occurs on the Gα subunit, one GPCR is capable to activate multiple G proteins. After dissociation, the free Gα-GTP and Gβγ subunits contribute to the regulation of "second messengers" that in turn control the activity

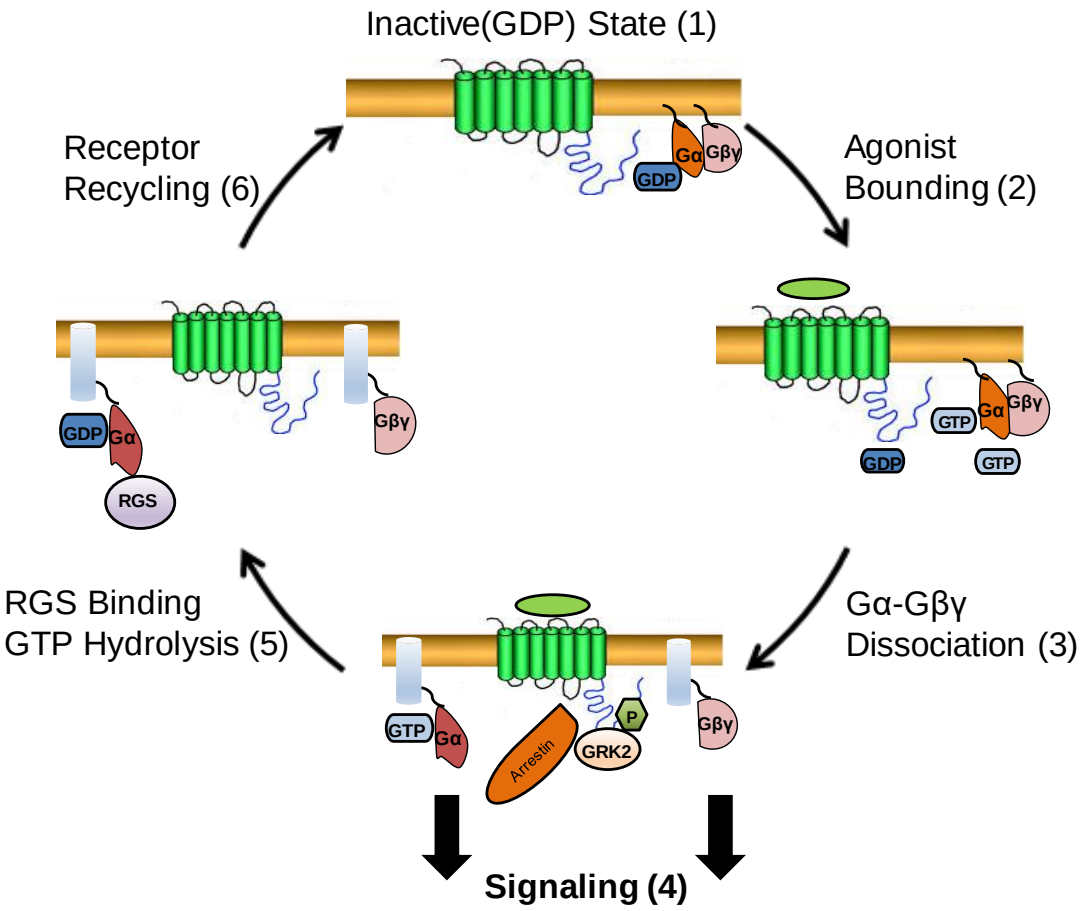
of protein kinases with role in intermediary metabolism (Fig. 1.1). For instance, the activation of $G\alpha_s$ is known to stimulate the production of adenylate cyclase that in turn upregulates cAMP formation and consequent protein kinase A (PKA) phosphorylation leading to smooth muscle relaxation and hence increases vasodilatation. Conversely, $G\alpha_i$ has a $G\alpha_s$-antagonistic effect culminating with reduced vasodilatation. On the other hand, $G\alpha_q$ activates phospholipase C (PLC) and induces vasoconstriction either via Ca^{2+} releases from intracellular stores or via protein kinase C (PKC) phosphorylation. Finally, $G\alpha_{12/13}$ is capable to activate the Rho kinase leading to smooth muscle contraction via myosin light chain phosphatase activity (MLCP) (Rhee, 2001; Noma *et al.*, 2006).

1.2.3 G Protein-Independent Signal Transduction

The known versatility of certain GPCRs to bind either directly to enzymatic effectors, or indirectly through adaptor proteins has prompted the idea that GPCRs might signal by coupling to non-G protein-regulated effectors. Such mechanisms would likely involve β-arrestins and alternative ternary complex formation for GPCR signaling (DeFea *et al.*, 2000; Luttrell *et al.*, 2001; Miller and Lefkowitz, 2001; Perry and Lefkowitz, 2002; Ahn *et al.*, 2003). Typical examples in this category are angiotensin antagonist [Sar1Ile4Ile8]-AngII binding to wild type AT_1 receptor (Luttrell *et al.*, 2001) or propranolol binding to β_2-adrenergic receptor (Azzi *et al.*, 2003), both leading to extracellular signal-regulated kinase (ERK1/2) activation in the absence of G-protein activation. While G proteins trigger a rapid and transient nuclear translocation of ERK phosphorylation, β-arrestin-mediated ERK activation is slower but more persistent and restricted to the cytoplasm (DeFea *et al.*, 2000; Luttrell *et al.*, 2001; Ahn *et al.*, 2004). Both transient and sustained activated

Figure 1.1: The schematic diagram of GPCR-G protein-effector model of GPCR signaling

At rest, the subunits the G protein complex are associated with each other while both receptor and the complex are not associated. Gαβγ complex is bound to GDP (1). Upon agonist activation, Gαβγ associates with the receptor, followed by GDP-GTP exchange (2). Then, Gαβγ dissociates from the receptor into a GTP- Gα subunit and Gβγ heterodimer leading to the activation of downstream signaling (3 & 4). Within seconds of agonist binding, GPCRs are phosphorylated by GRKs, followed by subsequent β-arrestin binding. β-arrestin bound receptors are prepared for endocytosis and sorted for either recycling or degradation. Simultaneously, RGS bind to the Gα subunit leading to GTP hydrolysis and resetting the receptor to its initial resting state (5 & 6).


Inactive(GDP) State (1)
Agonist
Bounding (2)
Gα-Gβγ
Dissociation (3)
RGS Binding
GTP Hydrolysis (5)
Receptor
Recycling (6)
Signaling (4)
GDP
Gα
Gβγ
RGS
GTP
P
GRK2
Arrestin

ERK1/2 pathways have been shown to regulate cell growth and differentiation (Sasagawa *et al.*, 2005).

1.2.4 Receptor Phosphorylation-Dependent Mechanism

If GPCRs are subjected to prolong activation, the receptor itself becomes a target for negative regulation. The processes responsible for the regulation of GPCR levels are typically divided into desensitization, internalization and downregulation. Collectively, these processes control the uncoupling of the receptor from G proteins, removal of receptors from plasma membrane followed by recycling or degradation, and reduced synthesis of new receptors. GPCR desensitization following agonist binding starts off with receptor phosphorylation that involves mainly the Ser and Thr residues (carboxyl-terminal tail and third intracellular loop) where the newly added large phosphates will interfere with G protein-coupling and will facilitate the recruitment of adaptor proteins with role in internalization (Ferguson and Caron, 1998; Lefkowitz, 1998; Kohout and Lefkowitz, 2003).

Phosphorylation of the receptor can be performed either heterologously via second messenger-dependent protein kinases (PKA or PKC) or homologously via GPCR kinases (GRKs) (Ferguson, 2007). Activation of second messenger-dependent protein kinases desensitize receptors that have not bound agonist thus agonist occupancy of the GPCR is not necessary for this process. In some situations, PKA phosphorylation can also alter the G protein-coupling selectivity and this will trigger the PKA-phosphorylated receptor to reverse the direction (Daaka *et al.*, 1997; Lawler *et al.*, 2001; Zamah *et al.*, 2002). The switch in coupling from $G\alpha_s$ to $G\alpha_i$ subunit might also tilt the balance towards alternate signaling pathways (Lefkowitz *et al.*, 2002).

Homologous desensitization involves phosphorylation of receptors that are in the agonist-occupied conformation. GRKs-mediated desensitization is a two-step process in which receptor phosphorylation is followed by arrestin binding. Furthermore, arrestin binding to receptor domains fulfils three main functions: to blocks GPCR-G protein interactions, to tag GPCR for chathrin-mediated endocytosis, and to couple GPCRs to G protein-independent signaling cascades. In this context, the primary role of GRKs in GPCR desensitization is to increase receptor affinity for arrestins, one example in this sense being GRK2-induced phosphorylation of β2 adrenergic receptor that increases by 10-30 fold its affinity for β-arrestin 1 (Lohse *et al.*, 1992).

1.2.5 Receptor Phosphorylation-Independent Mechanism

Since phosphorylation is not an absolute prerequisite for GPCR desensitization, phosphorylation-independent mechanisms also exist and they are typically mediated by GRKs (Pao and Benovic, 2002; Shenoy and Lefkowitz, 2003; Sterne-Marr *et al.*, 2004).

GRK2 can bind directly to free $G\alpha_{q/11}$ subunits through its RGS homology domain (Carman *et al.*, 1999). Crystallographic analysis has shown that GRK2 might be able to simultaneously interact with the receptor, free $G\alpha_{q/11}$, and Gβγ subunits (Lodowski *et al.*, 2003). Thereby, desensitization occurs as a consequence of Gβγ sequestration by receptor-bound GRK2 that hinders the re-association of receptor with GDP-bound $G\alpha_{q/11}$ (Dhami *et al.*, 2004).

1.3 GPCR Trafficking

1.3.1 Receptor Internalization

GPCR internalization or endocytosis – is a process slower than desensitization since it involves a period of few minutes after agonist binding. The internalization of the GPCRs may occur either in a constitutive or an agonist-stimulated manner (Ferguson, 2001). While endocytosis can take place via several different avenues, the most commonly employed one is mediated by clathrin. The two events that "prepare the field" for clathrin-dependent internalization are GRK-mediated GPCR phosphorylation and β-arrestin binding. The latter is usually regarded as one of the integral components of GPCR endocytosis, intracellular trafficking, and downregulation (Goodman *et al.*, 1996; Lefkowitz, 1998).

Depending on their pattern of interaction with β-arrestin isoforms, GPCRs can be grouped in two classes. "Class A" has a higher affinity for β-arrestin 2 than for β-arrestin1 and it has a transient interaction since upon internalization, the receptor-β-arrestin complex dissociates. Some of the most typical components of the "class A" are β_2 and α_1 adrenergic receptors.

On the other hand, "class B" receptors bind β-arrestin 2 and β-arrestin 1 with equal affinity and two of the representative members of this class are angiotensin AT_{1a} and neurotensin 1 receptors. While for class A receptor, the process of internalization is tightly connected to the lifecycle of an unstable receptor-β arrestin complex, class B receptors involve a stable receptor-β arrestin compound that internalizes as a unit to be subsequently processed by the endosomes (Barak *et al.*, 1997; Oakley *et al.*, 2000; Oakley *et al.*, 2001).

1.3.2 Early Endosomal Sorting and Trafficking

There are two mechanisms that are involved in the recycling pathway and the major contributor to the selection of one over the other is represented by the stability of the GPCR–β-arrestin interaction. Certain receptors, such as the β_2 adrenergic receptor that dissociates fast from β-arrestin, can return rapidly to the plasma membrane via recycling endosomes. On the other hand, when more stable bounds between receptors and β-arrestins are formed, such is – for instance – the case of AT_1R, recycling tends to be a much slower process that concludes with the degradation of the receptors (Pitcher *et al.*, 1995). The root cause of this behavior could be tracked down to β-arrestin dissociation that allows protein phosphatases to cleave receptor phosphates and thus enable its recycling. When dissociation does not occur – as is the case the case of stable complexes – resensitization will certainly be hindered (Ferguson and Caron, 1998; Ferguson *et al.*, 1998).

1.3.3 Receptor Recycling and Downregulation

Re-initialization of signal transduction via recycling of the internalized receptors is a more effective approach than the *de novo* receptor synthesis. Sorting of the GPCRs back into the recycling occurs either through a default mechanism via a "bulk" membrane flow or through a regulated process via Rab GTPases (Gruenberg, 2001; Dale *et al.*, 2004; Maxfield and McGraw, 2004).

The persistent loss of cell surface receptors – also known as downregulation - represents the least understood part of GPCR responsiveness. While the information stored at the transcriptional level is important, receptor internalization followed by either re-sensitization or degradation also plays a major role on the control of receptor density on the cell surface, particularly during the early stages of downregulation.

1.4 G Protein-Coupled Receptor Kinase Family

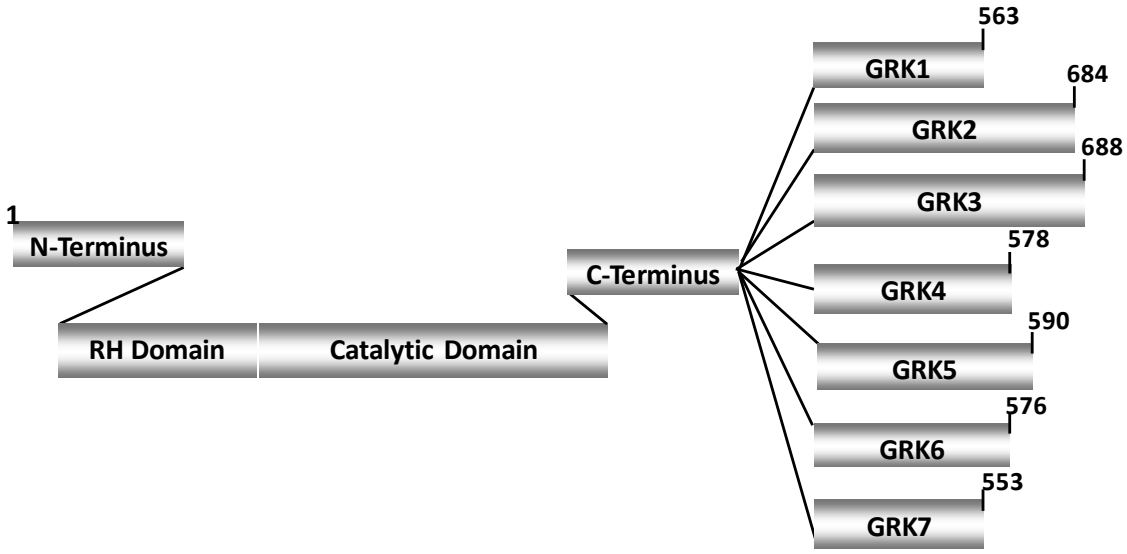
1.4.1 Structural Organization

There are seven genes involved in the encoding of the mammalian GRKs, a family of serine/threonine kinases with common structural and functional characteristics (Pitcher *et al.*, 1998a; Kohout and Lefkowitz, 2003). All GRKs share a similar basic structure that includes a well-conserved central catalytic domain (~ 270 aa) that is flanked by an amino terminal (~ 185 aa), and a carboxyl terminal with a variable length (~ 105-230 aa) (Penela *et al.*, 2003) (Fig. 1.2). The N-terminal domain plays an important role in receptor recognition (*e.g.*, the α-actinin-binding domain of GRK2) and intracellular membrane anchoring (*e.g.*, the calmodulin-binding domain of GRK5) (Palczewski *et al.*, 1993; Penn *et al.*, 2000). Furthermore, the amino terminus of GRK2 has been shown to be homologous to G-protein signaling at the $G\alpha_q/11$ binding RH domain and to inhibit GPCR-$G\alpha_q$ by sequestering the $G\alpha_{q/11}$ subunits. By contrast, the C-terminal domain contributes to their subcellular localization and agonist-dependent translocation by facilitating their interaction with lipids and other membrane proteins (Inglese *et al.*, 1992; Pitcher *et al.*, 1992; Koch *et al.*, 1993; Kohout and Lefkowitz, 2003; Penela *et al.*, 2003; Penela *et al.*, 2006; Reiter and Lefkowitz, 2006).

Based on the homology of their sequences, GRKs can be categorized into three main groups: rhodopsin kinase or visual subfamily (GRK1 and GRK7), the β-adrenergic receptor kinases subfamily (GRK2 and GRK3), and GRK4 subfamily (GRK4, GRK5 and GRK6). While certain common traits exist between all GRKs, each of them fulfils specific regulatory properties.

Figure 1.2: Linear diagram illustrating the three domain structures of GRK family

All GRK isoforms share a similar basic structure with a central catalytic domain, flanked by an amino terminal containing a regulated of G protein signaling (RGS) and a carboxyl binding domain of variable length. Aminoacid numbers – at the start and end of the structure – were shown to indicate the particular size of each isoform.


1
N-Terminus
RH Domain
Catalytic Domain
C-Terminus
563
GRK1
684
GRK2
688
GRK3
578
GRK4
590
GRK5
576
GRK6
553
GRK7

Furthermore, their anatomical localization is extremely diverse and inconsistent since GRK1 and GRK7 are expressed in retinal rods and cones, GRK4 is expressed in testis, cerebellum and kidney while GRK2, 3, 5, and 6 are ubiquitously expressed in mammalian tissues (Sallese *et al.*, 1997; Virlon *et al.*, 1998; Sallese *et al.*, 2000b).

With respect to their cellular localization, GRKs are mainly present in the cytoplasm or in the proximity of the plasma membrane. For instance, GRK1 and GRK7 are connected directly to the membrane due to their short COOH-terminal prenylation sequence that facilitates the attachment of lipid molecules acting as anchors to the cell membranes (Inglese *et al.*, 1992; Iaccarino *et al.*, 1998).

GRK2 and GRK3 are located in cytosol and bind within their C-terminus to the membrane-anchored Gβγ subunits of G protein complex (Pitcher *et al.*, 1992; Carman *et al.*, 2000). Since free Gβγ subunits are produced by ligand-bound receptors, the Gβγ will recruit the GRK2 and GRK3 isoforms. This will largely enhance the GPCR phosphorylation only when the fidelity of the desensitization process is critical in case of agonist-activated receptors. On the other hand, the specific interactions of GRK2 with the membrane-anchored Gβγ will also assist with the preservation of a membrane-bound GRK2 prior to the agonist-dependent GRK2 translocation (Penela *et al.*, 2003). However, in addition to their cytosolic localization, GRK2 was also found in mitochondria, even though this is a rather uncommon possibility (Fusco *et al.*, 2012; Chen *et al.*, 2013).

GRK4 and GRK6 possess both post-translational palmitoylation sites and lipid-binding positively charged elements that determine their constitutive membrane localization (Stoffel *et al.*, 1994; Premont *et al.*, 1996; Loudon and Benovic, 1997; Pitcher *et al.*, 1998a; Stoffel *et al.*, 1998). Both GRK4 and GRK6 have multiple splice-variant

forms that determine different structural domain organizations (Premont *et al.*, 1999; Vatter *et al.*, 2005). GRK5 is also found attached to the plasma membrane via its PIP2 binding domain even though lacks the classical palmitoylation site (Pronin *et al.*, 1998; Thiyagarajan *et al.*, 2004). One interesting case is that GRK6A splice variant includes elements that both promote and inhibit membrane localization. Furthermore, even though both cytoplasm and nucleus contain non-palmitoylated forms of GRK6A, its nuclear function remains unknown (Jiang *et al.*, 2007). However, nuclear localization sequences are common in all members of the GRK4 subfamily (Johnson *et al.*, 2004; Johnson *et al.*, 2013).

To a certain extent, the functional specificity of GRKs for a certain receptor can be explained through the differential subcellular targets and tissue expression levels that could be indeed one of the ways in which GRKs manage to accomplish different tasks in different tissues. However, a number of questions remain still open since variable expression levels of different GRK isoforms are unable to fully explain the receptor-kinase specificity. While receptor phosphorylation "bar coding" (Nobles *et al.*, 2011), cell-specific expression or structural characteristics have all emerged as viable explanations of the receptor specificity, the complete picture remains unclear.

1.4.2 Regulation of GRKs Activity and Mechanism of Action

The first and well described function of GRKs is the ability to phosphorylate active GPCRs (Wilden, 1995) and to allow binding of arrestins to block the cytoplasmic surface of receptors (Krupnick *et al.*, 1997) thus preventing their interaction with effector enzymes (Palczewski *et al.*, 1991; Chen *et al.*, 1993). The only exception from this overall functional

pattern of GRKs is GRK4α that was shown to be capable of phosphorylating the unstimulated GPCRs (Menard *et al.*, 1996; Rankin *et al.*, 2006).

GRKs were found to phosphorylate many other targets at the membrane as a response to receptor activation (Binder *et al.*, 1990; Binder *et al.*, 1996) that occurred with non-cognate pairs, as is the case of GRK2 that phosphorylates rhodopsin in a light-dependent manner (Benovic *et al.*, 1986). The shared activation characteristics allow few GRKs to be capable of phosphorylating virtually hundreds of GPCRs (Palczewski, 1997). The two conditions to be met for GRK-mediated phosphorylation of GPCRs are the presence of negatively charged lipids as well as the formation of a GRK docking pocket (inaccessible in the active state) in the cytoplasmic surface of the active receptor (Choe *et al.*, 2011; Rasmussen *et al.*, 2011a; Standfuss *et al.*, 2011).

In addition to GPCRs, GRKs are also capable of phosphorylating a broad variety of proteins such as single transmembrane domain tyrosine kinases (PDGFRβ), single transmembrane domain serine/threonine kinases, toll-like receptors, transcription factors and adapter proteins. The first non-GPCR protein identified was tubulin and both GRK2 and GRK5 were found to phosphorylate it (Carman *et al.*, 1998; Haga *et al.*, 1998; Pitcher *et al.*, 1998b).

However, GRKs were found to modulate cell functions that are independent of phosphorylation and this can be attributed mainly to their ability to interact with many signaling and trafficking proteins such as, actin (Freeman *et al.*, 1998), actinin (Freeman *et al.*, 2000), caveolin (Carman *et al.*, 1999; Gildea *et al.*, 2009), clathrin (Shiina *et al.*, 2001), Gα (Carman *et al.*, 1999), Gβγ (Pitcher *et al.*, 1992), PI3K (Naga Prasad *et al.*, 2001), tubulin (Carman *et al.*, 1998). In addition, GRKs modulate multiple cellular

responses such as cell migration (Penela *et al.*, 2008), cell survival (Chen *et al.*, 2013) and metabolism (Usui *et al.*, 2004; Cipolletta *et al.*, 2009; Ciccarelli *et al.*, 2011).

Many mechanisms have been proposed to explain the cytoplasmic regulation of GRKs. Even though it became soon clear that while a common component exists, kinase activity tends to be extremely specific to each GRK subtype. For instance, in HEK cells, PKA binds and phosphorylates GRK2 and this enhances Gβγ biding and promotes membrane translocation (Cong *et al.*, 2001). Similarly, PKC phosphorylation was found to augment GRK2 activity (Chuang *et al.*, 1995; Winstel *et al.*, 1996; Krasel *et al.*, 2001).

Because GRK2 is a short-lived protein that is polyubiquitinated and degraded in response to β2ARs (Penela *et al.*, 2001), it is natural to believe that transcriptional regulation might play an important role in GRK control, although very little is known about the mechanisms involved in the modulation of GRK mRNA levels. A different mechanism proposed for GRK2-specific regulation involves S-nitrosylation: when nitric oxide (NO) is abundant, this causes the inhibition of GRK2 activity towards GPCRs (Whalen *et al.*, 2007). Furthermore, GRK2 was found to have a bidirectional inhibitory relationship with endothelial NO synthase (eNOS) that is practically dependent on eNOS bioavailability (Huang *et al.*, 2013). GRK2 was also found to be phosphorylated by p38 protein (Liu *et al.*, 2013) and mitogen-activated protein (MAP) kinases (Pitcher *et al.*, 1999) that results in reduced phosphorylation of the active receptor and accentuated GRKs degradation (Elorza *et al.*, 2003; Chen *et al.*, 2013). However, phospholipids are also known to play a role in GRK activity modulation, typically via the PH domain of GRK2 and GRK3 (Onorato *et al.*, 1995; Pitcher *et al.*, 1996; Pronin *et al.*, 1998; Carman *et al.*, 2000).

1.4.3 GRKs and GPCR-Biased Signaling

The main mechanism responsible for GPCR downregulation is GRK-mediated phosphorylation followed by the binding of the β-arrestin (Premont *et al.*, 1995; Sterne-Marr and Benovic, 1995) acting as a clathrin adaptor with role in receptor internalization (Goodman *et al.*, 1996). However, it is possible that – prior to receptor downregulation – activated GPCRs become "biased" towards G protein or β-arrestin-mediated signaling. Furthermore, the pattern of receptor phosphorylation will determine a specific β-arrestin functionality according to the so-called "bar code" model (Nobles *et al.*, 2011). For instance, GRK6-mediated receptor phosphorylation leads to β-arrestin recruitment and activation of ERK1/2 signaling whereas GRK2-mediated phosphorylation determines signaling fading and/or receptor internalization (Kim *et al.*, 2005; Ren *et al.*, 2005; Kara *et al.*, 2006; Shenoy *et al.*, 2006; Jones *et al.*, 2007; Zidar *et al.*, 2009; Nobles *et al.*, 2011).

Another aspect of GPCR signaling is that some ligands that normally function as antagonists to block G protein signaling pathways may also function as agonists on alternative signaling pathways in the same cell (Galandrin *et al.*, 2007; Kenakin, 2007). This so-called "biased agonism" is particularly important with respect to β-arrestin signaling and is a target for the treatment of heart failure and hypertension. For example, it has been demonstrated that the β-blocker carvedilol, antagonizes the $G\alpha_s$-coupling of β_2ARs, but stimulates GRK2-mediated phosphorylation of the receptor, β-arrestin recruitment, and ERK1/2 phosphorylation (Wisler *et al.*, 2007; Lee *et al.*, 2008). This has led to the hypothesis that the unique efficacy of carverdilol in the treatment of heart failure may be related to the activation of β-arrestin-signaling (Wisler *et al.*, 2007). Biased agonism is not unique to $G\alpha_s$-coupled receptors since the AT_{1A}R antagonist

[Sar^1,Ile^4,Ile^8]Ang II fails to activate G protein signaling, but effectively promotes β-arrestin2-dependent ERK1/2 activation (Wei *et al.*, 2003; Rakesh *et al.*, 2010).

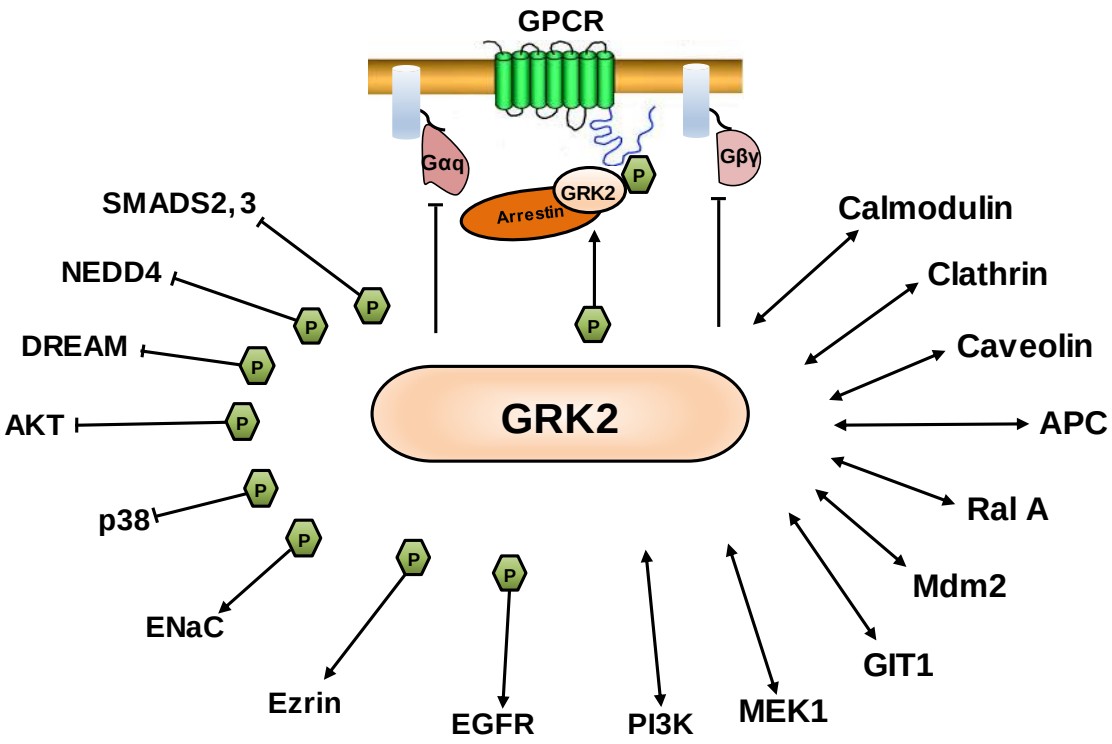
1.4.4 GRK2 and Hypertension

Among the seven GRKs, GRK2 seems to be the only isoform that is critical for the embryonic development (Jaber *et al.*, 1996), while gene ablation of the other kinases results in much more elusive phenotypes (Jaber *et al.*, 1996; Peppel *et al.*, 1997; Lyubarsky *et al.*, 2000; Gainetdinov *et al.*, 2003; Walker *et al.*, 2004). More importantly, the physiological functions of GRK2 extend beyond the embryonic development since this kinase has been found to be involved with cell cycle progression, migration, and differentiation (Kahsai *et al.*, 2010; Chen *et al.*, 2011). Moreover, the GRK2-mediated desensitization is also dependent on selective protein-protein interactions (Lodowski *et al.*, 2003; Tesmer *et al.*, 2005) that might occur outside of the cell membrane (Fusco *et al.*, 2012).

One of the interesting observations about GRK2 is that its N terminus has the ability to selectively interact with $G\alpha_q$. This interaction leads to sequestration of $G\alpha_q$ and further inhibition of $G\alpha_q$-mediated activation of PLC (Carman *et al.*, 1999; Sallese *et al.*, 2000a). The C terminus of GRK2 contains a pleckstrin homology (PH) domain capable of binding to phosphatidylinositol 4,5-biphosphate (PIP2) and free $G\beta\gamma$ subunits. The latter interaction is responsible for agonist-mediated translocation of GRK2 to the plasma membrane followed by the enhanced phosphorylation of the activated GPCRs. The C terminus of GRK2 also binds to clathrin and this leads to the internalization of certain GPCRs followed by their co-localization in endosomes along with GRK2 (Ruiz-Gomez and Mayor, 1997; Shiina *et al.*, 2001) (Fig. 1.3).

Figure 1.3: Schematic representation of the GRK2 interactions

In addition to its canonical role to phosphorylate active GPCRs, GRK2 can phosphorylate diverse non-GPCR substrates such as transcription factors and adaptor proteins. GRK2 also displays a complex network of functional interactions with signaling and trafficking proteins. (APC, Adenomatous polyposis coli protein; RalA, Ras like proto-oncogene A; Mdm2, Mouse double minute 2; GIT1, G protein-coupled receptor kinase-interacting protein; MEK1, Mitogen-activated protein kinase kinase 1; PI3K, phosphatidylinositol-3-kinases; EGFR, Epidermal growth factor receptor; Ezrin aka Cytovillin; ENaC, Epithelial sodium channels; p38 MAK, Mitogen-activated protein kinases; DREAM, Downstream regulatory element antagonist modulator; NEDD4, Neural precursor cell expressed developmentally down-regulated protein 4; SMADS3,4, Mothers against decapentaplegic homolog 3, 4;


GPCR
Gαq
Gβγ
GRK2
Arrestin
P
SMADS2, 3
NEDD4
DREAM
AKT
p38
ENaC
Ezrin
EGFR
PI3K
MEK1
GIT1
Mdm2
Ral A
APC
Caveolin
Clathrin
Calmodulin
GRK2

GRK2 was also shown to be involved in the negative modulation of the immune responses (Penela *et al.*, 2014), and this is accomplished by means of the phosphorylation-independent interactions that occurs with a large – and perhaps yet to be identified – number of proteins with roles in receptor internalization and signaling. For instance, GRK2 modulates MAPK signaling via direct association with MEK with further consequences on chemotactic responses (Jimenez-Sainz *et al.*, 2006).

Alterations in GRK2 expression and activity were found associated with a broad spectrum of pathologies including heart failure (Iaccarino *et al.*, 2005), multiple sclerosis (Vroon *et al.*, 2005), rheumatoid arthritis (Lombardi *et al.*, 2001). GRK2 levels in peripheral blood lymphocytes were positively correlated with impaired vascular GRK2 expression and hypertension (Gros *et al.*, 1997; Gros *et al.*, 1999; Gros *et al.*, 2000). Alterations in GRK2 expression and activity have been described in both human and animal models of hypertension. GRK2 overexpression in VSMCs *in vivo* results in mice that show impaired β-adrenergic-mediated vasodilatation, impaired cAMP accumulation and a modest increase in blood pressure (Eckhart *et al.*, 2002). However, vascular-specific knockdown of GRK2 expression(via genetic ablation or peptide inhibition) did not reduce hypertension even though β-adrenergic-mediated vasodilatation was improved (Cohn *et al.*, 2008).

Beyond GRK2, other GRKs were found to play complex and intricate roles with respect to blood pressure regulation. Vascular GRK5 overexpression in transgenic mice was associated with a hypertensive phenotype (Keys *et al.*, 2005). Low levels of GRK3 in human lymphocytes were associated with high blood pressure, a result that was also confirmed through a murine model (Oliver *et al.*, 2010).

On the other hand, overexpression of both GRK3 and GRK5 in HEK293 cells was found to desensitize dopamine D_1 receptors (Tiberi *et al.*, 1996), while inhibition of GRK6 decreased receptor desensitization (Fraga *et al.*, 2006). The levels of GRK6 in kidney were found lower in hypertensive rather than normotensive subjects.

To add to this picture, a growing body of work have suggested that GRK4 participates in the adverse responses observed in patients under antihypertensive medication (Bhatnagar *et al.*, 2009; Liu and Xi, 2012; Rayner *et al.*, 2012; Vandell *et al.*, 2012; Wagner *et al.*, 2012). This behavior is probably a consequence of the counterbalance that exists between the regulating role played by GRK4 on both dopamine-mediated natriuresis and renin-angiotensin system (RAS)-mediated antinatriuresis (Zeng *et al.*, 2008). GRK4 isoform is constitutively activated under basal conditions partially due to its capacity to bind to inactive $G\alpha_S$ and $G\alpha_{13}$ subunits (Keever *et al.*, 2008). So far, four splice variants were identified for GRK4 (Premont *et al.*, 1996) and all are expressed abundantly in human renal proximal tubules (Felder *et al.*, 2002; Villar *et al.*, 2009). GRK4 expression is also characterized by organ specificity, since its renal cortical expression was found increased in animal models of hypertension (Sanada *et al.*, 2006). Furthermore, the renal expression of GRK4 was found to be both mouse strain and salt intake-dependent (Escano *et al.*, 2009). Increased renal GRK4 activity was detected in hypertensive patients (Felder *et al.*, 2002) and alterations in various GRK4 gene variants were observed in subjects with different ethnic backgrounds (Bengra *et al.*, 2002; Zhu *et al.*, 2006; Venkatakrishnan *et al.*, 2013). Moreover, meta-analyses conducted on human studies revealed that while the GRK4-A486V gene variant is inversely correlated with hypertension among East Asians, it is positively associated with hypertension among Europeans.

1.5 Rab Family of Small GTPases

1.5.1 Structure of Rab Proteins

Rab proteins are the largest family of Ras superfamily of monomeric GTPases-binding proteins. Rab GTPases are small-molecular sized (21–25 kDa) proteins that are involved in many cellular functions including growth, trafficking, transduction, and fusion of membrane-bound organelles (Chavrier and Goud, 1999; Pereira-Leal and Seabra, 2000). Since their initial cloning in late 1980s, about 70 different members of Rab (*i.e.*, "Ras-like in rat brain") have been identified and characterized in humans (Pereira-Leal and Seabra, 2000; Zerial and McBride, 2001; Colicelli, 2004). Rab proteins enclose a compact, globular, GTP binding and hydrolysis domain that is linked to an unstructured C-terminus domain that tends to be the extremely diverse across Rab GTPase sequences (Itzen and Goody, 2011). Rab proteins undergoes conformational change when switching from the active, GTP-binding form to its inactive, GDP-binding counterpart. Mutations targeting GTP binding and hydrolysis are usually responsible for alter Rab4 function and thereby hinder the intracellular vesicular transport (Zerial and McBride, 2001).

1.5.2 Subcellular Localization and Microdomains

Rab proteins participates in the regulation of several endocytic, transcytic and exocytic transport pathways and they are commonly located to the cytoplasmic surface of membrane-bound organelles (Chavrier *et al.*, 1991; Ferro-Novick and Novick, 1993; Zerial and Stenmark, 1993; Ullrich *et al.*, 1996; Novick and Zerial, 1997). In addition to their predominant organelle-attached location, a minor fraction of each Rab protein is found in

cytosol in a complex with the guanine dissociation inhibitor (GDI) (Garrett *et al.*, 1993; Soldati *et al.*, 1993; Ullrich *et al.*, 1993).

While the majority of Rab proteins are expressed ubiquitously, some of them have a more specific tissue/cell type dissemination. For instance, Rab3 is expressed in the synaptic vesicles of neurons, Rab17 is localized in epithelial cells (Lutcke *et al.*, 1993), and Rab22 has been found mostly in hematopoietic cells (Hume *et al.*, 2001). The pathway regulated by Rab proteins is generally correlated with their subcellular location (Zerial and McBride, 2001). To exemplify, Rab6 and Rab8 were originally found to regulate the transport of newly synthetized membrane proteins (Huber *et al.*, 1993), while Rab4 and Rab15 seem to be involved with cargo transports (van der Sluijs *et al.*, 1992; Zuk and Elferink, 1999; McCaffrey *et al.*, 2001).

Rab proteins are characterized by domain structures in order to fulfill the spatial distribution of membrane proteins. The endosome domains are comprised of combinations of Rab proteins that dynamically change over time, but do not typically mix with each other. Therefore, three main endosomal domains were identified: one with Rab5 alone, one with combination of Rab4 and Rab5, and one containing Rab4 and Rab11 (Sonnichsen *et al.*, 2000; Zerial and McBride, 2001). These Rab populations have distinct biochemical and functional characteristics with specific pharmacological sensitivity. Generation of Rab endosomal domains depends not only on protein-lipid interactions (Simons and Ikonen, 1997; Mukherjee and Maxfield, 2000; Zerial and McBride, 2001), but also on the amount of energy generated during GTP hydrolysis which regulates the kinetics and limits the extension of effector recruitment (Zerial and McBride, 2001)

1.5.3 Rab Proteins in the Trafficking Pathway

Rab proteins cycle from their GDP-bound inactive to GTP-bound active forms between cytosol and membranes, respectively. This continuous cycle of activation, inactivation, and translocation is regulated by at least three key molecules: guanine nucleotide exchange factors (GEPs), GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs) (Pfeffer, 2005). Post-synthesis, Rab proteins associate with the cytosolic Rab escort protein (REP) to form a stable (GDP-bound) complex (Andres *et al.*, 1993; Wilson *et al.*, 1996).

The primary role of Rab proteins is to regulate each of the four major intracellular membrane trafficking steps: vesicle formation/budding, vesicle motility/delivery, vesicle tethering, and fusion of the vesicle membrane with that of the target compartment. Each of these different steps are being carried out with assistance from Rab effectors that bind to specific GTP-bound Rabs. Since a single Rab can interact with multiple effectors, it can be inferred that one Rab protein can regulate various biochemical reactions occurring at different sites along the pathway between two organelles.

Among all intracellular pathways, the endocytic one plays a key role during the transport and recycling of proteins. The functionality of the several Rabs located along the endocytic pathway has been characterized. The endocytic pathway works in a sequence of steps that start with the formation of clathrin-coated endocytic vesicles ("pits") to later become endosomes. Within them, the endocytosed components are then sorted to their different/final destinations involving either degradation or sorting (Gruenberg and Kreis, 1995; Gruenberg and Maxfield, 1995; Mellman, 1996). Different Rab proteins regulate each of the steps along the endocytic pathway (Chavrier *et al.*, 1990; Olkkonen *et al.*, 1993;

Ullrich *et al.*, 1996). For instance, Rab5 mediates trafficking from plasma membrane to early endosomes (Bucci *et al.*, 1992) while Rab4 mediates the fast recycling from early endosomes and recycling endosomes (van der Sluijs *et al.*, 1992; Daro *et al.*, 1996; Kouranti *et al.*, 2006) back to the plasma membrane.

Contrasting with endocytic pathways, the exocytic ones participate in the regulation of biosynthetic protein secretion and various Rab proteins are involved with different exocytic steps (Novick and Zerial, 1997). To exemplify, Rab1 and Rab2 govern trafficking from the endoplasmic reticulum (ER)-to-Golgi, Rab6 regulates the intra-Golgi traffic (Tisdale *et al.*, 1992), while Rab8 and Rab11 mediate the transport from the trans-Golgi network (TGN) to the surface (Huber *et al.*, 1993). Furthermore, the exocytic function of Rab11 seems to also be implicated in the development of the photoreceptor cells in embryos (Pelissier *et al.*, 2003; Satoh *et al.*, 2005; Giansanti *et al.*, 2007).

Newer theories have started to suggest that endosomes are in fact dynamic sites for the activation of GPCR-G proteins in a sense that RabGTPases were identified downstream of various signaling pathways and they influence gene expression and growth control. Along these lines, β_2AR-mediated induction of cAMP-dependent genes was found to be dysregulated by endocytic inhibitors (Tsvetanova and von Zastrow, 2014). Rab25 and Rab11a seem to participate in EGFR and TGFβ signaling as well as trafficking in the context of cell differentiation and proliferation (Lapierre and Goldenring, 2005; Nam *et al.*, 2010).

1.5.4 Rab4-Modulated Recycling of GPCRs

There are two isoforms associated with Rab4 protein, each of them being encoded by Rab4A and Rab4B genes (chromosomes 1 and 19). Rab4 isoforms are ubiquitously expressed and are characterized by a 93% homology, although their tissue expression is quite different. For instance, Rab4A is localized in the brain while Rab4B appears in B cells (Krawczyk *et al.*, 2007; Hoogenraad *et al.*, 2010).

With respect to its intracellular location, Rab4 resides in the endosomal system, in a sub-compartment with role in fast recycling to the plasma membrane (van der Sluijs *et al.*, 1992; Daro *et al.*, 1996), and only a relatively small (5%) amount was found in the Golgi complex. This latter localization suggests that Rab4 might also be involved in the traffic between endosomes and Golgi complex (de Wit *et al.*, 2001).

Neither the transport to the late endosomes nor the endocytic internalization are influenced in any way by the inhibitory Rab4-N121I mutant (Gerez *et al.*, 2000). Dephosphorylation of β_2AR occurs during the transit of the receptor between the Rab5- and Rab4-positive early endosomal compartments. While the overexpression of wild type Rab4 neither alters the rate of β_2AR nor accelerates the recycling of the receptor back to the surface of the cell, it appears that the overexpression of Rab4-N121I mutant is in fact capable to block β_2AR recycling and resensitization. In addition, the expression of either Rab4-N121I or wild type Rab4 does not prevent β_2AR phoshphorylation (Seachrist *et al.*, 2000).

The transgenic overexpression of Rab4 in mouse myocardium leads to significant increases of β-ARs in the plasma membrane as well as augments basal cAMP production in response to isoproterenol stimulation. In addition, the cardiac-specific overexpression of

Rab4 is also responsible for mild cardiac hypertrophy (Filipeanu *et al.*, 2006). Conversely, the cardiomyocyte-specific transgenic expression of an inhibitory Rab4-S27N mutant was found to reduce the β_2AR-regulated cardiac contractile function, primarily through βAR reallocation. Moreover, the Rab4-mediated recycling of internalized βARs is required for resensitization after agonist-binding as well as to maintain normal cardiac catecholamine responsiveness (Mohrmann and van der Sluijs, 1999; Somsel Rodman and Wandinger-Ness, 2000; Odley *et al.*, 2004).

Rab4 protein was found to co-localize with internalized AT_1R whose recycling is also controlled by Rab11 (Hunyady *et al.*, 2002; Seachrist and Ferguson, 2003; Dale *et al.*, 2004). As such, it was proposed that AT_1R recycling pathway is mediated by both Rab4 and Rab11 (Li *et al.*, 2008). The precise function(s) of Rab4 are still unclear, but it was speculated that it might facilitate the transported AT_1R vesicles to fuse with Rab11-positive perinuclear compartments, such that after fusion, Rab4 and Rab11 will form common recycling endosomes. Then, the AT_1R sorted for recycling will bud off via Rab11 and it will be eventually recycled back to the plasma membrane (Li *et al.*, 2008).

Dephosphorylation of AT_1R is facilitated by the overexpression of wild type Rab4 (but not Rab11), whereas a constitutively active Rab4-Q67L mutant will promote AT_1R resensitization (Esseltine and Ferguson, 2013). The association between various Rab GTPases and the AT_1R carboxyl-terminus typically leads to different functional outcomes. To exemplify, Rab5 is capable to promote the sequestration of AT_1R in early endosomes (Seachrist *et al.*, 2002), Rab7 has a role in the trafficking of the AT_1R to lysosomes (Dale *et al.*, 2004), while Rab4 was found to promote the resensitization of the AT_1 receptor. Even though Rab11 can co-immunoprecipitate more effectively with AT_1R, Rab4 is in fact

capable to displace Rab11. Therefore, it could be inferred that small differences in Rab4 expression might translate into dramatically different alterations in AT_1R activity.

1.6 Pathogenesis of Hypertension

Hypertension or high blood pressure represents a complex, multifactorial disease that constitutes a major cause of morbidity and mortality worldwide affecting more than 1 billion people (Lawes *et al.*, 2008). Recent statistics estimate that 19 in 20 Canadians will develop hypertension over an average life span (Public_Health_Agency_of_Canada, 2010). Even though high blood pressure might not have immediate negative health consequences, its increased values over prolonged periods of time leads to deterioration of a number of vital organs such as heart, brain, or kidney. The associated pathologies – cardiac hypertrophy and failure, stroke, nephropathy – were identified as root causes of the increased morbidity and mortality associated with chronic hypertension (No_authors_listed, 1967). While current therapeutic approaches focused on blood pressure reductions are generally capable to avert the progression of the pathologies associated with hypertension (No_authors_listed, 1967; No_authors_listed, 1979; No_authors_listed, 1985), extensive data collected suggest that hypertensive treatments have reduced blood pressure to target levels in less than 50% of cases . Despite significant improvements in the effectiveness of hypertensive treatments, the incidence of uncontrolled or resistant hypertension remains high. Under these circumstances, a better understanding of the molecular and cellular mechanisms responsible for the pathogenesis of hypertension represents one of the priority avenues to be pursued towards the development of improved therapeutic strategies.

When it comes to the etiology of hypertension, several major hypotheses have been proposed and among them the "mosaic theory" of hypertension seems to be the prevailing dogma. Over the last decades ongoing research attempted to determine which one of the "myriad" of alterations in blood pressure regulatory systems occurs earliest in the process and which ones are critical in the maintenance of the hypertensive state.

One of the favored hypothesis asserts that the arteriolar injury is primary and this increases peripheral resistance, leading to cardiac hypertrophy and kidney strain (Gull and Sutton, 1872). This initial idea was later refined to add that reduction in the luminal diameter of small vessels is the hallmark of blood pressure increases that are associated with idiopathic hypertension (Folkow, 1987).

Another hypothesis revolved around for a key role for the kidney in pathogenesis of hypertension (Johnson, 1872). The primary "culprit" of this hypothesis is the "pressure natriuresis" mechanism whose dysfunctionality leads to hypertension (Borst and Borst-De Geus, 1963; Guyton *et al.*, 1972). According to the theory proposed by Guyton *et al.* (1972), the majority of systems causing elevation in blood pressure would only induce temporary increases, whereas the prolonged ones require important alterations of the pressure natriuresis curve. Subsequent studies on this topic have largely supported this hypothesis (Hall *et al.*, 1990; Cowley and Roman, 1996; Wang *et al.*, 2000). Further endorsements of the renal involvement in hypertension came from a series of kidney transplantation studies performed both on human patients (Curtis *et al.*, 1983) and animal models (Bianchi *et al.*, 1974; Dahl and Heine, 1975; Rettig *et al.*, 1990). In this regard, when kidneys from normotensive, salt-resistant rats were reciprocally transplanted into hypertensive rats characterized by salt-sensitivity, the blood pressure was either

normalized (in case of the hypertensive recipients) or increased (in case of the normotensive recipients) (Bianchi *et al.*, 1974; Dahl and Heine, 1975; Kawabe *et al.*, 1978). Similar blood pressure normalization patterns were also observed in hypertensive patients who received kidneys from normotensive donors (Curtis *et al.*, 1983). The primary role of the kidney is also further reinforced by the genetic studies that have established correlations between the disorders associated with abnormal blood pressure and fluid reabsorption along the nephron (Lifton *et al.*, 2001). Evidently, the larger emphasis placed on the kidney in the context of the hypertension does not exclude the role of non-renal mechanisms that could intervene either by means of AT_1R-mediated vasoconstriction (Crowley *et al.*, 2005) or systemic nervous system (Naraghi *et al.*, 1994). There are a number of vasoactive systems that have been suggested as key contributors to the hypertensive process, namely: increases in intrarenal vasoconstrictors (*e.g.*, angiotensin II (Navar *et al.*, 2003), endothelin-endothelin A receptor (Noll *et al.*, 1996)) or reductions in intrarenal vasodilators (*e.g.*, kallikrein–bradykinin (Ardiles *et al.*, 2003), endothelin–endothelin B receptor (Kohan, 2006), dopamine (Hermann *et al.*, 2006; Katori and Majima, 2006; Jose *et al.*, 2010).

A long standing debate argued that hypertension is in fact determined by a complex of genetic and environmental factors ("mosaic hypertension") because an increased frequency of hypertension was observed in families of index patients (Page, 1967).

Another hypothesis proposed that neural mechanisms are a major contributor to hypertension. Following the initial idea that the activation of sympathetic nervous system leads to the onset of essential hypertension, a number of subsequent studies have shown that patients with activated sympathetic nervous system (SNS) exhibit an increased basal

cardiac pace, elevated blood pressure in response to stimuli and increased catecholamine levels (Julius and Schork, 1978; Goldstein, 1983; Mancia *et al.*, 1999). Multiple mechanisms appear to be involved in this neural hypothesis, including faulty autoregulation of the baroreceptors (Mancia *et al.*, 1999), increased hypothalamic responses (Mancia *et al.*, 1997), stimulation of renal sympathetic leading to the activation of the adrenergic pathways for the central nervous system (Campese and Park, 2006) or increased thoracolumbar sympathetic activity (Smithwick, 1949).

Despite the century-long research on the molecular basis of hypertension, it is clear that – similar with other chronic pathologies – the picture is still unclear and far from being complete. While the long-term regulation of blood pressure is undoubtedly the outcome of a collaborative/overlapping activity between neuronal, renal and circulatory systems, with few exceptions (Joyner *et al.*, 2008; Coffman, 2011; Charkoudian and Wallin, 2014), the majority of the studies tend to (over)emphasize one of the three components on the expense of the other two. In this context, the terms that have been coined for the two major theories in the field are neurocentric (Navar, 2010) and renocentric (Jelakovic and Mayer, 1995; Joyner *et al.*, 2008). According to the former one, the overactivity of the SNC is involved in both initiation and maintenance of the blood pressure in patients with essential hypertension (DeQuattro and Miura, 1973; Esler *et al.*, 1977; Grassi, 2010; Fisher and Paton, 2012; Esler, 2014; Mancia and Grassi, 2014; Grassi *et al.*, 2015). By contrast, the renocentric supporters argue that while the kidney is the main long-term regulator of blood pressure, the nervous system plays an important role in short term regulation (Borst and Borst-De Geus, 1963; Guyton and Coleman, 1969; Coffman and Crowley, 2008; Brands, 2012; Hall *et al.*, 2012; Coffman, 2014; Ivy and Bailey, 2014).

1.6.1 Adrenergic System and Vascular Tone

The sympathetic nervous system plays a key role in the regulation of peripheral vascular resistance and capacitance of the vascular system. Peripheral resistance reflects a net balance between vasoconstrictor and vasodilator mechanisms. As part of this systemic activity, vascular tone is mediated by G-protein-coupled receptor signaling pathways. Previous studies support the assumption that hypertension is in part due to dysregulation and desensitization of GPCRs linked to vasodilation (Feldman and Gros, 1998; Feldman and Gros, 2006; Ferguson and Feldman, 2014).

The major mechanism mediating the hormonal regulation of vasodilatation is via the activation of one or more of the nine known subtypes of adrenergic receptors of which β – adrenergic receptors (βAR) are the prototype. While the β_1A receptors are only present in certain smooth muscle cells such as in coronary vessels, β_2-adrenergic receptors are express in the most vascular cells and their action involves both endothelial- and vascular smooth muscle-mediated regulation of vascular tone (Orlov *et al.*, 1996).

One of the key contributors to the regulation of the blood pressure is constituted by the vascular smooth muscle (VSM) in a sense that the hypercontractive state of VSM is usually regarded as the hallmark of essential hypertension. Alterations in β_2-adrenergic receptors lead to reduced adenylyl cyclase activity and cAMP formation that in turn prevents VSM relaxation (Kamikawa *et al.*, 1980; Feldman *et al.*, 1987; Anand-Srivastava *et al.*, 1991). Interestingly, $G\alpha_i$ (a major inhibitor of adenylyl cyclase) was found upregulated in VSM and myocardium of hypertensive humans and animal models. β_2AR may couple to $G\alpha_i$ and potentially switch the signal leading to activation of alternative regulatory pathways (Feldman and Gros, 2006).

1.6.2 Renin Angiotensin System and Vascular Tone

One of the most powerful regulators of blood pressure is the renin-angiotensin system (RAS) (Kobori *et al.*, 2007; Navar *et al.*, 2011). From a clinical perspective, it has been established that RAS is a key regulator of blood pressure and fluid balance, particularly since the untimely activation of RAS – that is quite common in renal artery stenosis – has been linked with profound cardiovascular morbidity and hypertension (Lonn *et al.*, 1994). In addition, studies performed on patients with essential hypertension who do not have typical signs of RAS activation have shown that angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are capable to successfully diminish blood pressure, suggesting that dysregulation of RAS was responsible for the increased in blood pressure (Hansson *et al.*, 1999; Yusuf *et al.*, 2000; Dahlof *et al.*, 2002).

From a mechanistic perspective, RAS is a potent modulator of the renal pressure-natriuresis balance and this directly affects the characteristics of the blood pressure levels in both normal and pathologic conditions (Hall, 1986; Hall *et al.*, 1999). In this regard, while the chronic infusion of Ang II shifts the pressure-natriuresis curve to the right – meaning that RAS activation requires higher blood pressures for a certain amount of sodium excretion – the administration of ACE inhibitors has an opposite effect, essentially meaning that the excretion of the sodium in urine is facilitated at lower blood pressures levels (Hall, 1986).

1.6.2.1 Components of the Renin Angiotensin System

The functionality of RAS relies on a multi-enzymatic cascade of processes according to which angiotensinogen (AGT) is sequentially converted, by means of a two-step enzymatic reaction, into Ang II. Renin is a key-rate limiting enzyme whose expression/secretion is

controlled by a renal baroreceptor mechanism working in conjunction with sodium chloride concentration to the macula densa (Carey *et al.*, 1997). However, in addition to its regulatory role in RAS activity-renin through its pro-renin and renin receptors expressed by renal glomeruli as well as vasculature-stimulates both pro-fibrotic and pro-inflammatory pathways independently of Ang II (Nguyen and Muller, 2010).

Through a "feed-forward" mechanism, Ang II has the potential to augment the angiotensinogen synthesis in the kidney with impact on RAS activity. Simultaneous upregulation of intrarenal AGT at both mRNA and protein levels is present in multiple animal models of Ang II-mediated hypertension (Schunkert *et al.*, 1992; Kobori *et al.*, 2001; Kobori *et al.*, 2007; Gonzalez-Villalobos *et al.*, 2008). The increase in the intra-renal AGT levels can be reversed by means of AT_1 receptor blockers, suggesting that the activation of AT_1R has a promoting role that in turn accelerates the progression of the hypertensive disease. The long-term increases in systemic or renal Ang II levels will stimulate the hypertensive pathogenic factors, including growth factors, pro-inflammatory cytokines, oxidative and mechanical stress (Ruiz-Ortega *et al.*, 2002) and in turn, they will all cooperate to boost the expression of angiotensinogen in kidney. Thus, the urinary angiotensinogen level might be used as a clinical indicator of the renal RAS activation in hypertensive patients (Kobori *et al.*, 2009; Konishi *et al.*, 2011).

1.6.2.2 Role of Renal RAS

A growing body of research has shown that in addition to its core functionality as RAS-mediator (Bader *et al.*, 2001), Ang II can induce glomerular growth and sclerosis (Mezzano *et al.*, 2001). Furthermore, increased levels of Ang II in the kidney are associated with albuminuria and elevated glomerular collagen deposition (Muller *et al.*, 2002). Several

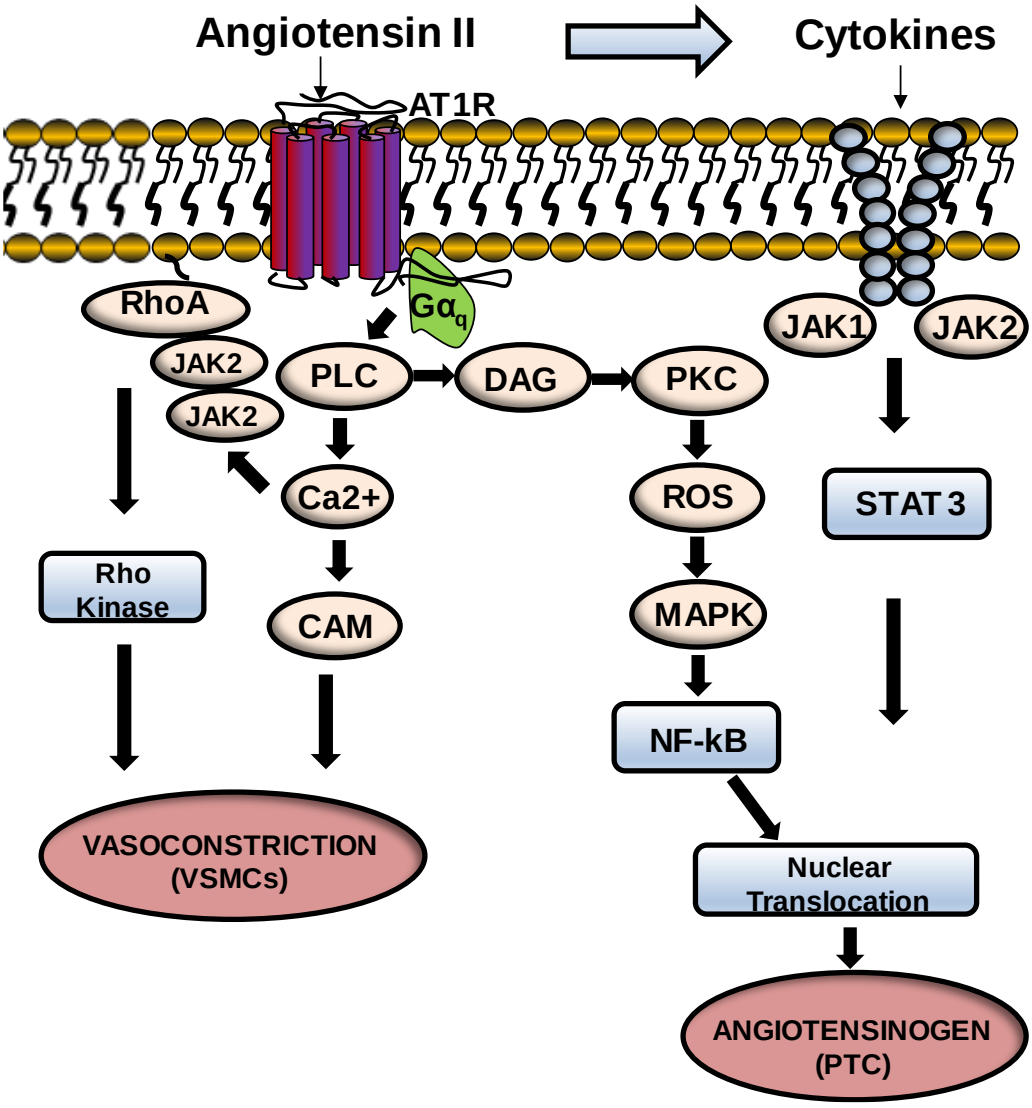
studies have suggested that blocking RAS would slow down or even stop the progression of renal fibrosis (Brewster and Perazella, 2004). Treatment with the ARB-losartan was able to regress the pathologic increases of collagen I and IV gene/protein expressions (Boffa *et al.*, 2003), while the administration of ACE inhibitor-enalapril was associated with reversions of the tubulointerstitial and glomerulosclerosis (Adamczak *et al.*, 2003).

Angiotensin II was also known to modulate immune responses largely by inducing the expression of pro-inflammatory cytokines that in turn can potentiate renal injury (Crowley *et al.*, 2008). Previous studies have shown that AT_1 receptors as well as other RAS components are present in several mononuclear cell populations with immunomodulatory roles (Nataraj *et al.*, 1999; Jurewicz *et al.*, 2007). To further reinforce the strong link between the immune and angiotensin system, Muller *et al.* (2002) have shown that immunosuppression is capable to reduce inflammatory cell infiltration in the kidney, renal structural damage, and albuminuria in the Ang II-induced hypertension.

Previous studies have shown that AT_1 receptor plays a critical role in mediating the Ang II-dependent mechanism of hypertension. AT_1R is a member of the GPCR family capable to couple with $G\alpha_{q/11}$ and $G\alpha_i$ (Higuchi *et al.*, 2007). As shown in Fig. 1.4, once Ang II activates AT_1R, a cascade of events is being triggered. As a result, different second messengers are activated via G protein-dependent pathways and this further translates into vasoconstriction induction, changes in gene transcription, promotion of cell growth and migration, etc. (Touyz and Berry, 2002; Higuchi *et al.*, 2007; Mehta and Griendling, 2007; Miyata *et al.*, 2014). The activation of AT_1 receptors are responsible for the internalization of the ligand-receptor complex that in turn triggers intracellular Ang II increases (Zou *et al.*, 1996; Zou *et al.*, 1998; Zhuo *et al.*, 2002).

Figure 1.4: Schematic of the proposed mechanisms of Ang II-mediated hypertension

Ang II-activated AT_1Rs trigger the activation of a cascade of downstream second messengers that further translate into vasoconstriction and changes in gene transcription. As explained in the text, increased levels of Ang II indirectly lead to accumulation of intrarenal cytokines and synergistically add to increase proximal tubular (PT) angiotensinogen expression levels.


Angiotensin II
Cytokines
AT1R
RhoA
JAK2
JAK2
PLC
DAG
PKC
Gαq
JAK1
JAK2
Ca2+
ROS
STAT 3
Rho Kinase
CAM
MAPK
NF-kB
Nuclear Translocation
VASOCONSTRICTION (VSMCs)
ANGIOTENSINOGEN (PTC)

Approximately half of the intra-renal increases in Ang II levels are due to AT_1R-mediated uptake of circulating Ang II (Higuchi *et al.*, 2007; Shao *et al.*, 2009). The AT_1 receptor blockade leads to important increases in glomerular filtration rate, renal blood flow and profound increases in sodium excretion. Taken together, all these observations demonstrate the canonical role played by the activated intra-renal AT_1 receptors in the regulation of renal function, electrolyte and body fluid homeostasis, and blood pressure homeostasis.

1.7 Hypothesis and Specific Objectives

GPCR activity represents a delicate, but coordinated balance between molecular mechanisms governing: receptor signaling, desensitization, and resensitization (Ferguson, 2001). However, these processes do not occur in isolation, rather they occur simultaneously with each cycle of receptor-ligand interaction and the patterns of desensitization and resensitization differ between GPCR subtypes. It is now clear that alterations in the balance between GPCR signaling, desensitization and resensitization are associated with both cardiac failure and hypertension (Ungerer *et al.*, 1993; Gros *et al.*, 1997; Feldman and Gros, 1998; Gros *et al.*, 2000; Wu *et al.*, 2001; Eckhart *et al.*, 2002; Odley *et al.*, 2004; Harris *et al.*, 2007; Raake *et al.*, 2008).

Over the past years, our group has identified many molecular mechanisms underlying differences in GPCR signaling, desensitization and resensitization in heterologous cell culture systems (Oakley *et al.*, 1999; Zhang *et al.*, 1999; Anborgh *et al.*, 2000; Oakley *et al.*, 2000). This includes the GRK2-and β-arrestin-dependent and -independent regulation of GPCR desensitization and endocytosis (Ferguson *et al.*, 1995; Ferguson *et al.*, 1996; Zhang *et al.*, 1997; Dhami *et al.*, 2002; Dhami *et al.*, 2004; Dhami *et al.*, 2005), along with the role of Rab GTPases in regulating the trafficking of GPCRs

between distinct intracellular membrane compartments (Seachrist *et al.*, 2000; Seachrist *et al.*, 2002; Dale *et al.*, 2004; Esseltine *et al.*, 2011).

Alteration in GRK2 expression has been noticed in both cardiac failure and hypertension, and GRK2 overexpression in VSMCs *in vivo* results in mice that show a modest increase in blood pressure. By contrast, the consequence of reduced GRK2 activity on vascular function has not been addressed. Previous studies indicate that Rab4 expression is increased in cardiac hypertrophy and failure. However, the contribution of Rab4 to the regulation of GPCR activity in VSMCs and hypertension has not been analyzed.

We **hypothesize** *that alterations in both GRK2 and Rab4 expression/activity modulate vascular GPCRs signaling and thereby play an essential role in the mechanism of hypertension.* Specifically, the **aims** of this thesis are to:

1. **Assess the molecular alterations that contribute to onset of hypertension in a mouse model of GRK2 inhibition.**
2. **Determine whether GRK2 inhibition leads to alteration of renal function and stimulation of renal-mediated mechanisms of hypertension.**
3. **Examine whether modulation of Rab4GTPase activity influences vascular GPCRs signaling and blood pressure homeostasis.**

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CHAPTER 2:

GRK2 TARGETED KNOCK-DOWN RESULTS IN SPONTANEOUS HYPERTENSION AND ALTERED VASCULAR GPCR SIGNALING

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2.1 Introduction

Blood pressure homeostasis and vascular reactivity is controlled via a complex network of cell signaling mechanisms. Among the broad network of receptors and signaling molecules regulating blood vessel reactivity, members of the GPCR family are known to play a central role. Since GPCRs expressed on vascular smooth muscle cells (VSMCs) fine tune and maintain the balance between constriction and relaxation of blood vessels, their modulation has been a primary target in hypertension. Indeed, approximately one third of the cardiovascular drugs used in current clinical practice directly target GPCR signaling and function in one way or the other (Siryk-Bathgate *et al.*, 2013). GPCRs function to modulate vascular tone by transducing hormonal signals into changes in intracellular second messenger levels, effector enzymes and channel activity (Lefkowitz, 2013).

Agonist activation of GPCR signaling is counteracted by intrinsic cellular mechanisms that turn off or dampen the agonist-generated signal, a phenomenon referred to as receptor desensitization or G protein-uncoupling. This provides an essential physiological "feedback" mechanism that protects against both acute and chronic GPCR over-stimulation. The classical paradigm for GPCR desensitization involves phosphorylation of agonist activated receptors by G protein-coupled receptor kinases (GRKs), which promote the binding of β-arrestin proteins that function both to uncouple the receptor from the G protein and target receptors for clathrin-dependent endocytosis and internalization (Goodman *et al.*, 1996; Krupnick and Benovic, 1998; Laporte *et al.*, 1999; Ferguson, 2001). GRK-mediated phosphorylation followed by β-arrestin binding not only contributes to GPCR desensitization and endocytosis, but also couples GPCRs to the activation of G protein-independent receptor signals (Gesty-Palmer and Luttrell, 2008).

GRK2 is ubiquitously expressed in the body, regulates the vast majority of GPCRs, and partakes in several cellular processes like cell cycle progression, migration and differentiation. The GRK2 RH domain comprises a $G\alpha_{q/11}$ binding site that binds specifically to $G\alpha_{q/11}$ subunit (Carman *et al.*, 1999; Ferguson, 2007). Consequently, it has been demonstrated that $G\alpha_{q/11}$-coupled GPCR signaling can be attenuated in a GRK2 phosphorylation-independent manner via the association of the GRK2 RH domain with $G\alpha_{q/11}$ (Dhami *et al.*, 2002; Dhami *et al.*, 2004; Willets *et al.*, 2004; Iwata *et al.*, 2005).

From a mechanistic perspective, alterations in GRK2 expression and activity have been detected in both human and animal models of hypertension (Gros *et al.*, 1997; Gros *et al.*, 1999; Gros *et al.*, 2000; Eckhart *et al.*, 2002; Harris *et al.*, 2007; Cohn *et al.*, 2008; Izzo *et al.*, 2008; Cohn *et al.*, 2009; Morris *et al.*, 2010; Napolitano *et al.*, 2012; Avendano *et al.*, 2014). Specifically, GRK2 protein expression is elevated in lymphocytes from hypertensive patients and is correlated with reductions in the β-adrenergic receptor (βAR)-stimulated vasodilation (Gros *et al.*, 1997). The increased GRK2 expression might be an important prerequisite in the impairment of βAR-mediated vasodilation associated with hypertension, especially since the increases in lymphocyte GRK2 are paralleled by those in vascular GRK2 (Gros *et al.*, 2000). Moreover, increases in systolic blood pressure in humans correlate well with increases in GRK2 mRNA expression, but not with either GRK3 or GRK5 levels (Cohn *et al.*, 2009). Cardiac overexpression of GRK5 results in marked β_2AR desensitization, whereas GRK2 overexpression in cardiac muscle leads to significant impairment of Ang II-mediated cardiac contractility (Rockman *et al.*, 1996). Thus, it is possible that GRK2 may preferentially regulate the signaling of $G\alpha_{q/11}$-coupled GPCRs due to the fact that GRK5 does not have a functional RH domain (Ferguson, 2007).

GRK2 may have the unique capacity to block $G\alpha_{q/11}$-coupled GPCR signaling via its interaction with $G\alpha_{q/11}$. Consequently, alterations in GRK2 expression are proposed to induce a shift in the balance between vasoconstrictor and vasodilator mechanisms suggesting that GRK2 may function as a potential molecular mediator contributing to the induction of hypertension.

Given the important role played by GRK2 in regulating both phosphorylation-dependent and -independent GPCR desensitization and endocytosis, as well as β-arrestin -biased cellular signaling (Jaber *et al.*, 1996; Zhang *et al.*, 1997; Luttrell and Kenakin, 2011), the primary goal of this study was to clarify the link between changes in GRK2 expression and altered vascular GPCRs signaling.

We have successfully derived a line of viable shGRK2 transgenic mice with significant reduction of GRK2 protein expression in the aorta, heart, liver and kidneys. These mice are intrauterine growth retarded, are spontaneously hypertensive and have impaired vascular reactivity. In addition, VSMCs cultured from shGRK2-knockdown mice demonstrate reduced $G\alpha_s$- and $G\alpha_{q/11}$ GPCR desensitization, altered extracellular regulated kinase 1/2 (ERK1/2) and Akt signaling with age, as well as age-dependent increases in cellular proliferation and migration in response to AT_1, but not β_2AR, activation. Our data indicate that the loss of GRK2 expression shifts the balance of GPCR-regulated vasodilator and vasoconstrictor mechanisms in the favor of $G\alpha_{q/11}$ that at least in part could be responsible for the hypertensive phenotype observed in shGRK2 transgenic mice.

2.2 Experimental Procedure

2.2.1 Development and Characterization of shGRK2 Transgenic Mice

The shGRK2 transgenic mouse strain was generated using a U6 RNA polymerase III promoter to drive ubiquitous GRK2-shRNA AATCTTTGACTCCTATATTAT (shGRK2) expression and was subcloned HindIII/BamHI into the pSilencer 2.0-U6 vector. A 766 bp fragment containing both the U6 promoter and shGRK2 was excised by digestion with PvuII and microinjected into B6C3F1 mouse embryos that were transferred to pseudo-pregnant recipients. After weaning offspring were examined for integration of the genomic DNA by polymerase chain reaction (PCR) analysis and positive offspring backcrossed onto a C57Bl/6 background for 10 generations. Animals were housed in an animal care facility in cages of 2 or more animals, and were maintained on a 12-hour light/12-hour dark cycle at 24°C. Mice received food and water *ad libitum*. The experimental protocol involving animals was approved by the Animal Use Subcommittee of Western University, according to the guidelines of the Canadian Council on Animal Care.

2.2.2 Assessment of Blood Pressure

Blood pressures and heart rates were recorded from conscious mice at 4-5, 8, 12-14 and 24-25 weeks old, using the non-invasive CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA). To assess the possible influence of anxiety on blood pressure, non-invasive tail-cuff blood pressures and heart rates were obtained in 12-week old anesthetized (ketamine/xylazine 100/10 mg/kg intraperitoneal) mice. Invasive arterial blood pressures and heart rates were recorded in 12-week old anesthetized (ketamine/xylazine) mice after cannulating the right carotid artery with a Millar-tip

transducer catheter (model SPR-261, 1.4F; Millar Instruments, Inc., Houston TX). To assess *in vivo* vasoconstrictor responsiveness, mice were injected with 1 mg/kg phenylephrine intraperitoneal and blood pressures were recorded.

2.2.3 Indirect Calorimetry, Activity and Inactivity

Assessment of metabolic and activity parameters were obtained using the Comprehensive Laboratory Animal Monitoring System (CLAMS) metabolic chamber (Columbus Instruments, Columbus, OH, USA).

2.2.4 Vasomotor Studies

Vascular reactivity was assessed in second-order mesenteric arteries obtained from 12-14 week old wild-type and shGRK2 mice were mounted on a pressure myograph (Living Systems Instrumentation, St. Albans, Vermont). Passive and active vessel diameter perfusion pressure relationships (20-120 mm Hg) were obtained. The set-point was defined as the lowest perfusion pressure at which significant myogenic constriction was first observed. Extent was defined as the magnitude of the percent myogenic tone at a given perfusion pressure, and strength was defined as the slope of the active diameter-pressure relationship. Contractile responses to phenylephrine (1 nmol/L to 10 μmol/L) or KCl (10 to 120 mmol/L) (Sigma-Aldrich, Oakville, ON, Canada) were recorded in vessels pressurized to 60 mm Hg. To examine Ca^{2+} sensitivity, vessels were washed twice in Ca^{2+}-free physiological salt solution (1 mmol/L EGTA) and placed in Ca^{2+}-free physiological salt solution (PSS) containing 120 mmol/L KCl. Contractile responses to increasing extracellular $CaCl_2$ (0.5 to 3.0 mmol/L) were recorded. Vasodilatory response to isoproterenol (1 nmol/L to 10 μmol/L) or sodium nitroprusside (0.1 nmol/L to 10 μmol/L)

were performed in 60 mm Hg pressurized vessels pre-contracted with 3 μmol/L of phenylephrine.

2.2.5 H&E and Connective Tissue Staining of Aorta

Paraffin-fixed aortic tissues were sectioned (8μm) and placed on positively-charged microscope slides. After deparaffinization and rehydration through serial decreases of ethanol concentration (100%, 95%, and 70%), slides were subjected to a general H&E staining protocol. Collagen deposition was visualized by means of Masson's trichrome staining (Trichrome Stain-Kit, Abcam, Cambridge, MA, USA) and Movat penthachrome staining (Penthachrome Stain Kit, Nordic BioSite, Plymouth Meeting, PA, USA).

2.2.6 Primary VSM Cell Culture

Vascular smooth muscle cells (VSMCs) were isolated by enzymatic digestion from thoracic aortas of 4-12-24 week old GRK2 shRNA transgenic and age-matched C57Bl/6 male mice and maintained in primary culture for a subsequent number of 7 passages. Cells were grown as a monolayer in Dulbecco's Modified Eagle Medium (Life Technologies, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (BioShop, Burlington, ON, Canada), 50 μg/ml PDGF-B (Sigma-Aldrich, Oakville, ON, Canada) in a humidified incubator with 5% CO2 at 37^{o}C.

2.2.7 Cyclic Adenosine Monophosphate (cAMP) - Glo Assay Analysis

The cAMP GLO assay (Promega, Fitchburg, WI, USA) was carried out according to the manufacturer's protocol. VSMCs were seeded into a Poly-D-lysine coated, white, clear-bottom 96-well plates (~10.000 cells per well) and incubated overnight. Cells were treated with increasing concentrations of agonists for 15 min and lysed with cAMP-GLO Lysis

Buffer. Then, cAMP-GLO detection solution containing protein kinase A was added for 20 min, followed by the addition of Kinase-Glo reagent. Luminescence was measured using a Victor plate reader (PerkinElmer Life Sciences, Billerica, MA, USA).

2.2.8 Fura - 2 Florescence Ca^{2+} Imaging

VSMCs grown on 25 mm round glass cover ships were loaded with 5 µM Fura-2 acetoxymethyl ester (Invitrogen, Mississauga, ON, Canada) at 37 C for 20 minutes in Krebs Ringers buffer (pH = 7.4). Cells were then washed and incubated for another 10 minutes in Krebs Ringers buffer. Fura-2 dual excitation and emission was accomplished using 340- and 380-nm excitation filter and a Photon Technology International DeltaRam V imaging system. The coefficients required for $[Ca^{2+}]$ calculations were calibrated by means of the method described by Grynkiewicz *et al.* (1985).

2.2.9 Western Blot Analysis

VSMCs were plated at $2x10^5$/well in 6 well plates. Next day, cells were serum starved by culturing them in serum free medium for 48 hours. Prior to the protein extraction, cells were incubated in the absence (negative control) and presence of either isoproterenol (10µM), angiotensin II (100nM) or with their biased agonists carvedilol (10µM) and SIIAngII (100µM) over) over different time intervals (5, 15, 30, 60, 90, 120, 150, and 180 minutes). For preparation of the protein lysate, cells were treated with M-PER® Mammalian Protein Extraction Reagent supplemented with HALT Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA). Fifteen micrograms of total protein were electrophoresed per well on a 10% SDS-polyacrylamide gel and transferred onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA, USA). Immunoblotting was performed with the following primary

antibodies: monoclonal anti-actin and smooth muscle α-actin antibody, p44/p42 (ERK1/2) rabbit polyclonal antibody, phospho-p44/p42 (ERK1/2) rabbit polyclonal antibody, phospho-Akt (Ser 473) rabbit monoclonal antibody, Akt (pan) mouse monoclonal antibody (Cell Signaling Technology) diluted in a solution consisting of 3% BSA in TBST (20mM tris-base, 0.14M NaCl, 0.01% Tween, pH 7.4) overnight at 4^{o}C. Blots were probed afterwards with a mixture of IRDye polyclonal secondary antibodies (1:10000) (LI-COR Biosciences, Lincoln, NE, USA) for one hour. Images were read with an Odyssey infrared imaging system and the average density of each band was quantified using the Image Studio software (LI-COR Biosciences, Lincoln, Nebraska USA). For quantitative analysis of the protein levels, the average densities of target bands were quantified using the application software of the Odyssey infrared imaging system. For each condition, the band density of analyzed protein was normalized to the band density of the corresponding total protein (total-ERK1/2, AKT) or loading control (GADH).

2.2.10 Boyden Chamber Assay Analysis

VSMCs migration was assessed with Boyden chambers using Transwell® inserts (Corning Life Sciences, Oneonta, NY, USA)). Polycarbonate membrane inserts with 8 μm pore opening placed within 24-well plates were used. A two hundred microliter suspension of serum-starved cells at a concentration of $2x10^5$/ml was added in the upper chamber while various concentrations of agonists were added to serum-free media in the lower chamber. The assembled chamber inserts were then incubated at 37°C, 5% CO_2 for 24 hours. VSMCs were then fixed with methanol and stained with eosin/thiazine. Direct microscopic counting at 40X magnification (Leica DFC 295, Leica Microsystems, Germany) of cells

that have migrated to the lower side of the membrane was performed and a mean value for each sample was calculated.

2.2.11 Trypan Blue Exclusion Assay

VSMCs at $2x10^5$/ml were seeded into T25 flasks for 48 and 96 hours and then harvested with Trypsin-EDTA combined with media, spun down, and suspended with Trypan Blue (1:1). In order to generate a growth curve, an automated cell counter (Invitrogen, Burlington, ON, Canada) was used to calculate total cell number, live cells, dead cells, and viability.

2.2.12 Cell Proliferation ELISA, BrdU Assay Analysis

Serum-starved VSMCs cells to a final concentration of $2x10^5$ cells/ml were seeded onto 96-well tissue-culture microplates, treated with different type of agonists for 24 hours, and a cell proliferation ELISA BrdU (colorimetric) assay (Roche Applied Science, Indianapolis, IN, USA) was performed according to manufacturer's instruction. For signaling studies, serum starved VSMCs were pretreated with various concentrations of PI3 kinase inhibitor (LY294002) (Cell Signaling Technology, Danvers, MA, USA), Akt inhibitor (Akti-1/2) (Abcam, Cambridge, MA, USA), and MEK 1/2 inhibitor (U0126) (Cell Signaling, Danvers, MA, USA). After incubation, the reaction product was measured with a plate reader, Infinite M200 (TECAN, Morrisville, NC, USA) at wavelength of 370 nm.

2.2.13 Statistical Analysis

Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA, USA). All parametric data were analyzed with one-way Analysis of Variance followed by Tukey-Kramer or Dunnett post-hoc comparisons. When

two data sets were compared, a Student's t-test was used. Statistically relevant differences between mean values were determined based on $p < 0.05$ criterion.

2.3 Results

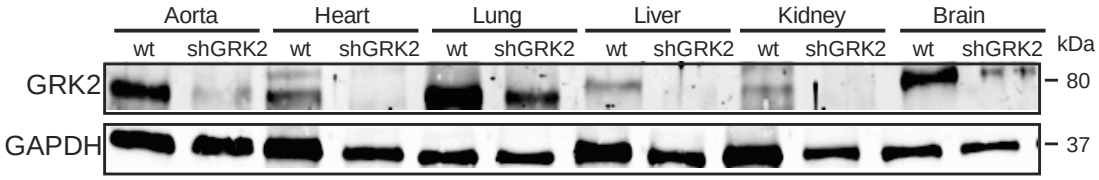
2.3.1 shGRK2 Mice Are Smaller and Have Reduced Body Weight

We employed shRNA, using a U6 mouse polymerase III promoter as a transgene, to universally knockdown, but not knockout, GRK2 protein expression in transgenic mice. The GRK2 shRNA transgene incorporated into the Y chromosome resulting in only viable transgenic male mice. Thus, age-matched C57BL/6 male mice were used as controls. The resulting shGRK2 transgenic mice had reduced GRK2 protein expression in all tissues tested including: the aorta, heart, lungs, liver, kidneys, and brain (Fig. 2.1A). Our time-course observation also documents a significant decreased in GRK2 mRNA expression level in aortic arteries of shGRK2 mice when compared with age-matched control C57BL/6 mice (Fig. 2.1B). Male shGRK2 transgene positive pups at E17.5 were significantly smaller in size and had lower body weight as compared to female *in utero* wild-type littermates (Fig. 2.1C and 1D). Despite the smaller body weight at E17.5 for shGRK2 positive male pups, the percentage of male versus female pups at this stage was equal. At the time of weaning (4 weeks of age), we observed that the percentage of male versus female was significantly lower suggesting a significant post-natal loss of shGRK2 mice (Fig. 2.1E). The lower body weight of shGRK2 male mice was still apparent at both 12 and 24 weeks of age (Fig. 2.1F). Similarly, assessment of body length (nose to tip of tail) revealed that shGRK2 mice are significantly smaller in body size at 12 weeks of age (shGRK2: 16.5 ± 0.1 cm, n=6 vs. C57Bl/6: 18.1 ± 0.1 cm, n=5, $p<0.05$).

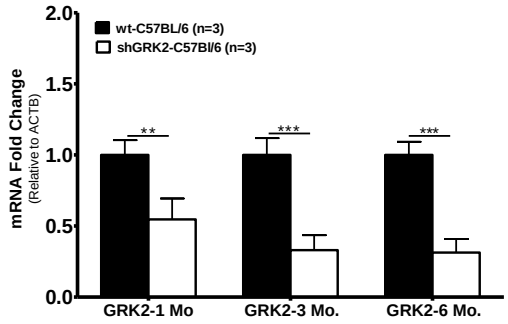
Figure 2.1: Characterization of shGRK2 transgenic mice

(A) Western blot was used to validate GRK2 protein expression in a variety of tissues isolated from C57Bl/6 and shGRK2 transgenic mice. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for differences in protein expression. **(B)** Quantitative RT-PCR analysis of GRK2 mRNA expression in aortic tissues isolated from C57Bl/6 and shGRK2 mice. Expressions levels are normalized to actin (ACTB). **(C)** Digital image of wild-type female and male shGRK2 pups outlining the difference in body size. **(D)** Quantification of body weight measurements for wild-type female, male shGRK2 and male C57Bl/6 pups at E17.5 days (n=20). (**E)** Proportion of male shGRK2 and female wild-type mice at E17.5 (female, n=22; male, n=20) and 4 weeks of age (female, n=108; male, n=44). **(F)** Quantification of body weight measurements in male mice at 3 (n=13) and 6 months (n=6) of age. * Indicates statistical significant differences versus female littermate ($p < 0.05$). ** Indicates statistical significant differences versus C57Bl/6 male mice ($p < 0.05$).

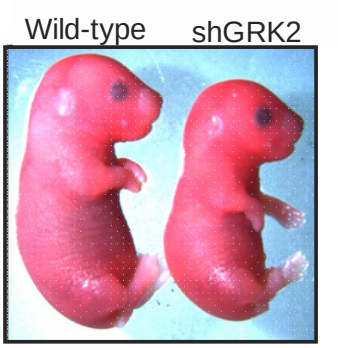
A)


Aorta
Heart
Lung
Liver
Kidney
Brain
wt
shGRK2
kDa
GRK2
GAPDH
80
37

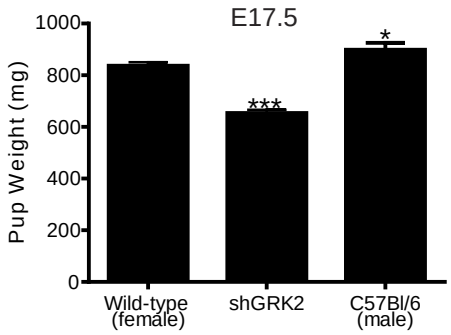
B)


wt-C57BL/6 (n=3)
shGRK2-C57Bl/6 (n=3)
mRNA Fold Change
(Relative to ACTB)
2.0
1.5
1.0
0.5
0.0
**
***
***
GRK2-1 Mo
GRK2-3 Mo.
GRK2-6 Mo.

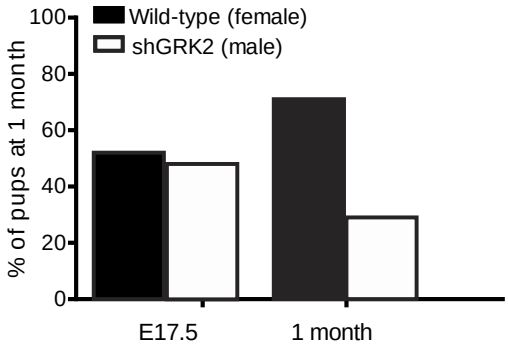
C)


Wild-type
shGRK2

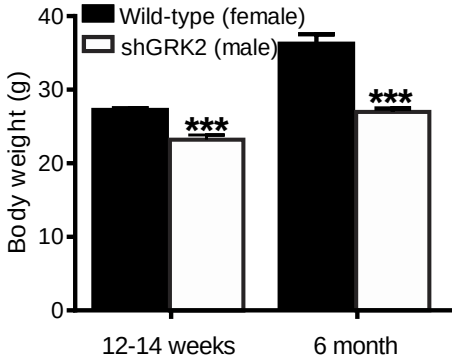
D)


E17.5
Pup Weight (mg)
1000
800
600
400
200
0
***
*
Wild-type
(female)
shGRK2
C57Bl/6
(male)

E)


Wild-type (female)
shGRK2 (male)
% of pups at 1 month
100
80
60
40
20
0
E17.5
1 month

F)


Wild-type (female)
shGRK2 (male)
Body weight (g)
40
30
20
10
0
***
***
12-14 weeks
6 month

2.3.2 Metabolic Parameters, Activity and Inactivity Are Altered in shGRK2 Mice

We assessed indirect calorimetry, activity and inactivity measurements in both 12-week old shGRK2 and wild-type C57Bl/6 mice using metabolic cages. No significant differences in O_2 consumption, CO_2 production, respiratory exchange ratio, energy expenditure, and food or water consumption between shGRK2 and wild-type mice were observed during the light cycle (Fig. 2.2A-F). However, both O_2 consumption and CO_2 production were significantly increased in shGRK2 mice during the dark/active cycle (Fig. 2.2A and 2.2B). Interestingly, assessment of inactivity (sleep) and activity revealed that shGRK2 mice had a significant reduction in their sleep time during the dark/active cycle, as well as a significant increase in both total and ambulatory activity measurements (Fig. 2.2G-I).

2.3.3 shGRK2 Mice Develop Hypertension with Age

No significant differences in systolic and diastolic blood pressure or heart rate were observed between shGRK2 and wild-type mice at 4-5 weeks of age using the non-invasive tail-cuff method (Fig. 2.3A). Interestingly, systolic blood pressure was significantly higher in 8-week old shGRK2 mice as compared to age-matched wild-type mice (Fig. 2.3B). Hypertension was established in 3 month-old shGRK2 mice as compared to wild-type controls with significant increases in both systolic and diastolic blood pressures (Fig. 2.3C). The elevated blood pressures were still evident in shGRK2 mice at 6 months of age (Fig. 2.3D). To eliminate the possibility of a stress/anxiety-induced elevation in blood pressure, we obtained blood pressures and heart rates in lightly anesthetized mice using the tail-cuff methods and both systolic and diastolic blood pressures were significantly elevated in shGRK2 mice (Fig. 2.3E). We also assessed hemodynamic parameters via invasive Millar catheterization and found that both systolic and diastolic

Figure 2.2*:* Assessment of metabolic parameters, activity and inactivity in shGRK2 transgenic mice

Representative graphs of different parameters from 3 month-old wild-type and shGRK2 mice monitored by means of metabolic cages showing: **(A)** volume of O_2, **(B)** volume of CO_2, **(C)** respiratory exchange ratio, **(D)** energy expenditure/heat, **(E)** food, **(F)** water, **(G)** sleep, **(H)** total activity, and **(I)** ambulatory activity. Data is represented as the mean ± SD of 8 mice in each group. *Indicates significant differences as compared with C57Bl/6 mice ($p < 0.05$).

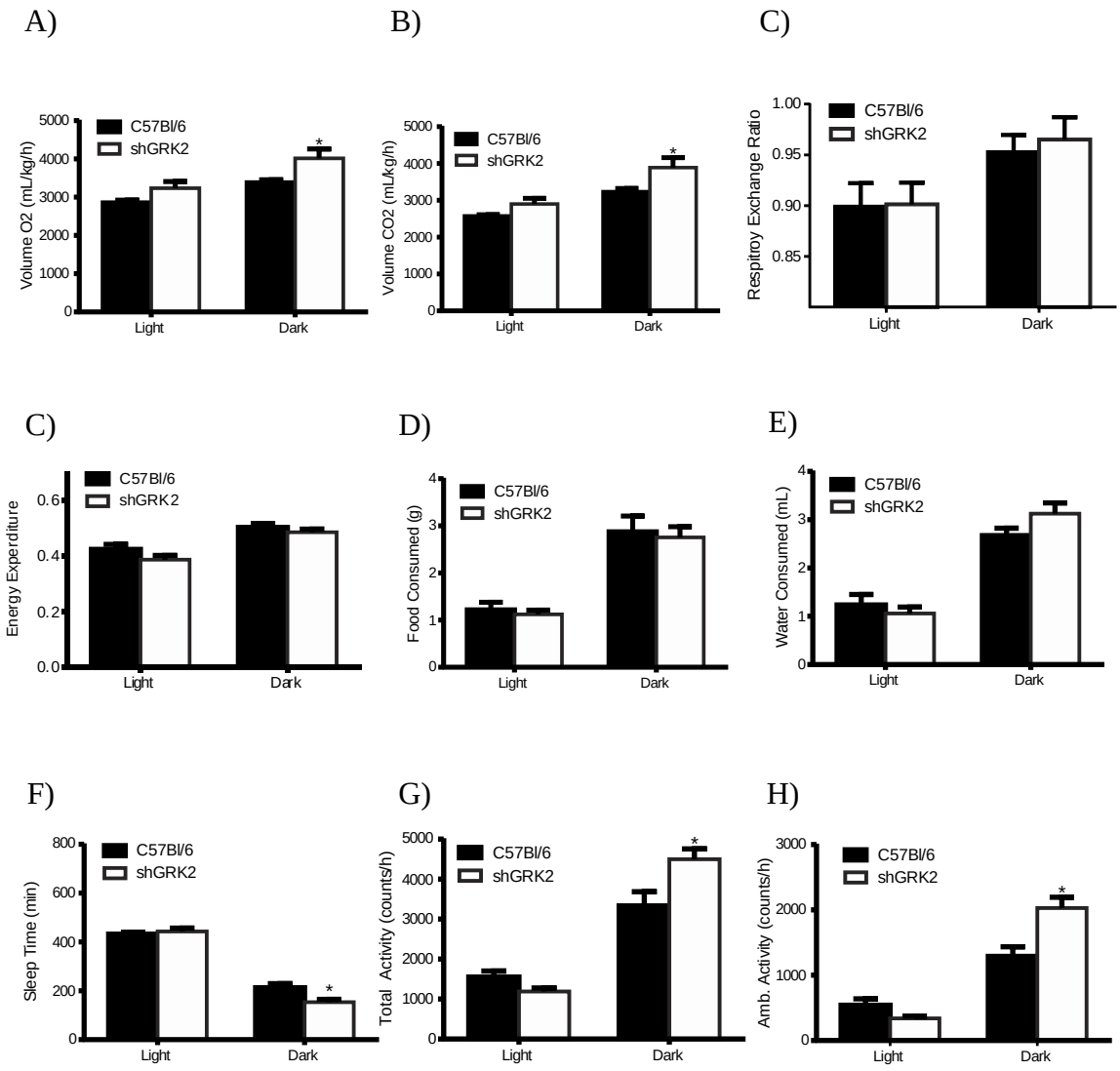
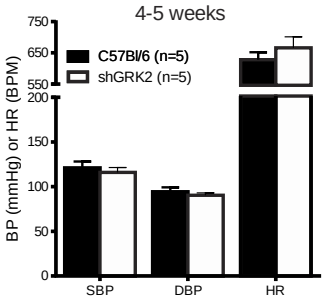

A)
B)
C)
Volume O2 (mL/kg/h)
Volume CO2 (mL/kg/h)
Respitroy Exchange Ratio
C57Bl/6
shGRK2
Light
Dark
C)
D)
E)
Energy Experditure
Food Consumed (g)
Water Consumed (mL)
F)
G)
H)
Sleep Time (min)
Total Activity (counts/h)
Amb. Activity (counts/h)

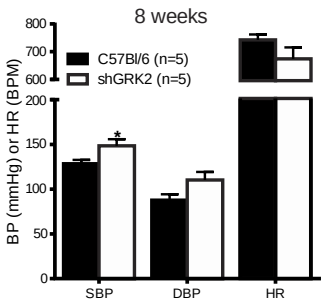
Figure 2.3*: Assessment of blood pressures and heart rates in shGRK2 transgenic mice*

Blood pressures and heart rates of C57Bl/6 and shGRK2 mice, as measured by non-invasive tail cuff CODA method at **(A)** 4-5 weeks, **(B)** 8 weeks, **(C)** 3 months and **(D)** 6 months of age. **(E)** Blood pressures and heart rates in lightly anesthetized 3 month-old mice using the tail-cuff method. **(F)** Blood pressures and heart rates measured via invasive Millar catheterization at 3 months of age. **(G)** Blood pressure responsiveness in Millar cannulated 3 month-old mice after phenylephrine administration. Data are represented as the mean ± SD of 5 independent experiments. * Indicates statistically significant differences versus C57Bl/6 mice ($p < 0.05$).

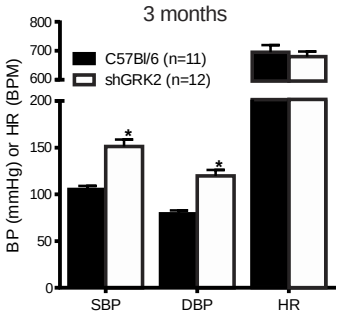
A)


4-5 weeks
C57Bl/6 (n=5)
shGRK2 (n=5)
BP (mmHg) or HR (BPM)
SBP
DBP
HR

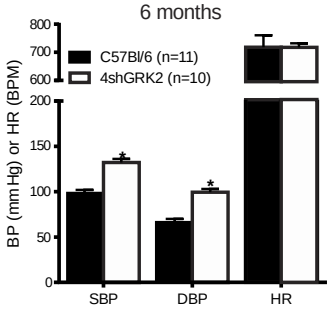
B)


8 weeks
C57Bl/6 (n=5)
shGRK2 (n=5)
BP (mmHg) or HR (BPM)
SBP
DBP
HR

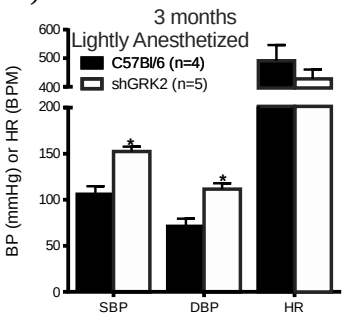
C)


3 months
C57Bl/6 (n=11)
shGRK2 (n=12)
BP (mmHg) or HR (BPM)
SBP
DBP
HR

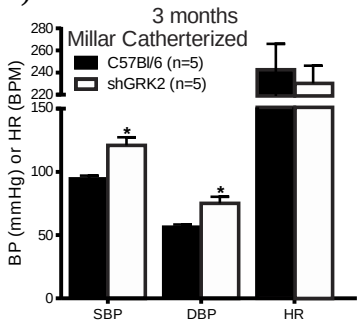
D)


6 months
C57Bl/6 (n=11)
4shGRK2 (n=10)
BP (mmHg) or HR (BPM)
SBP
DBP
HR

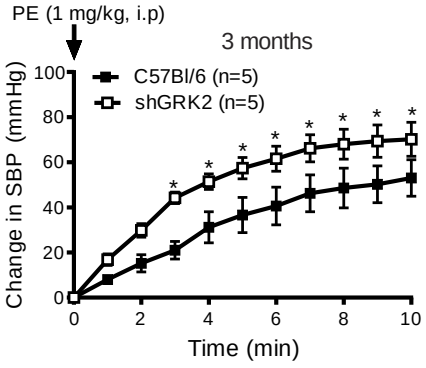
E)


3 months
Lightly Anesthetized
C57Bl/6 (n=4)
shGRK2 (n=5)
BP (mmHg) or HR (BPM)
SBP
DBP
HR

F)


3 months
Millar Catherterized
C57Bl/6 (n=5)
shGRK2 (n=5)
BP (mmHg) or HR (BPM)
SBP
DBP
HR

G)


PE (1 mg/kg, i.p)
3 months
C57Bl/6 (n=5)
shGRK2 (n=5)
Change in SBP (mmHg)
Time (min)

blood pressures were significantly higher in shGRK2 mice as compared to wild-type controls (Fig. 2.3F). In Millar cannulated mice, phenylephrine (PE, 1 mg/kg) injection significantly elevated systolic blood pressure in both wild-type and shGRK2, but the change in systolic blood pressure was significantly higher in shGRK2 mice as compared to wild-type controls (Fig. 2.3G).

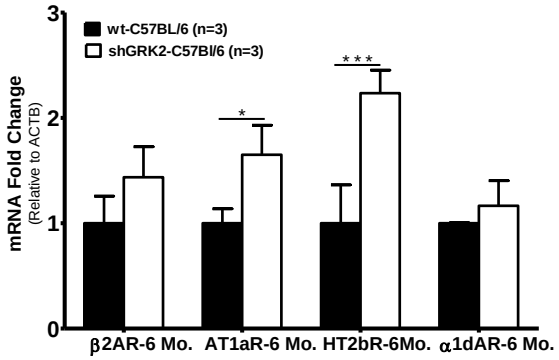
2.3.4 GRK2 Deficiency Results in Altered Vascular Reactivity

To assess the role of GRK2 inhibition in the regulation of GPCR expression, the mRNA levels of several GPCRs were measured. shGRK2 seems to have a stimulatory effect on GPCRs with role in vasoconstriction such as AT_1R, α_1AR, and 5HT-2bR as well as on vasodilatatory β_2A receptors (Fig. 2.4A). Mesenteric arteries from shGRK2 mice demonstrated enhanced myogenic tone development with a significant increase in the extent of myogenic constriction (Fig. 2.4B). Additionally, the set-point of the myogenic response was significantly reduced in mesenteric arteries from shGRK2 mice when compared to wild-type controls (80 vs. 100 mmHg, $P < 0.05$) (Fig. 2.4B). Similarly, we observed a significant increase in the contractile responses to phenylephrine in arteries obtained from shGRK2 mice (EC_{50}: 85.2 ± 12.8 nM, n = 5) when compared to wild-type controls (EC_{50}: 247 ± 25 nM, n = 5) (Fig. 2.4C). To determine if the increase in receptor-mediated vasomotor response was generalized to all constrictor agents, we examined the response to increasing doses of potassium chloride (KCl). No significant differences in KCl-mediated constriction were observed between shGRK2 and wild-type mouse arteries (Fig. 2.4D). To determine whether the enhanced vasomotor responses observed in arteries from shGRK2 mice was attributable to heightened Ca^{2+} sensitivity of the contractile

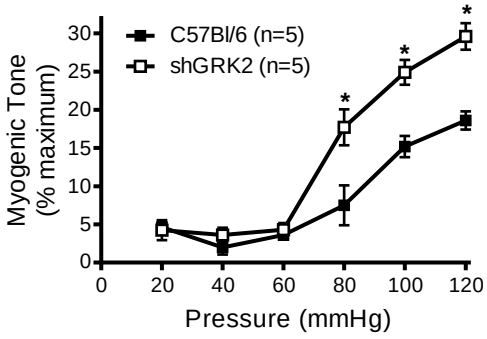
Figure 2.4: Assessment of vascular reactivity in mesenteric arteries obtained from wild-type and shGRK2 mice

(A) Quantitative RT-PCR analysis of several GPCRs mRNA expression in aortic arteries. Expressions levels are normalized to ACTB. **(B)** Myogenic responses of second-order mesenteric arteries collected from 3 month-old C57Bl/6 and shGRK2 mice as assessed by pressure myography. Contractile responses of mesenteric arteries to **(C)** phenylephrine, **(D)** potassium chloride and **(E)** calcium chloride. Vasodilatatory responses of mesenteric arteries to **(F)** isoproterenol and **(G)** sodium nitroprusside. Data points are represented as the mean ± SD. * Indicates statistically significant differences versus C57Bl/6 ($p < 0.05$).

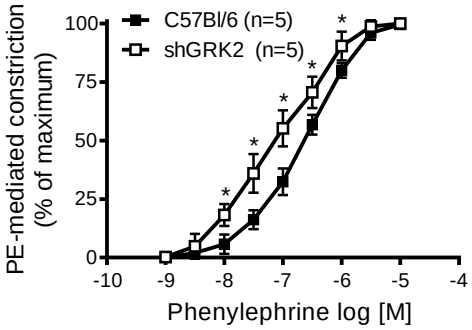
A)


wt-C57BL/6 (n=3)
shGRK2-C57Bl/6 (n=3)
mRNA Fold Change
(Relative to ACTB)
β2AR-6 Mo.
AT1aR-6 Mo.
HT2bR-6Mo.
α1dAR-6 Mo.

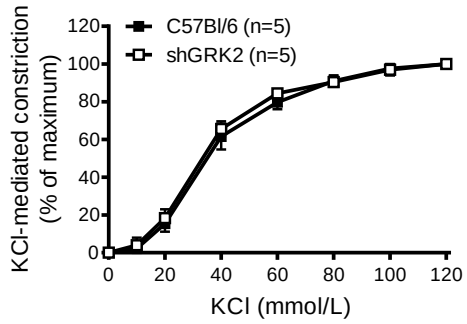
B)


C57Bl/6 (n=5)
shGRK2 (n=5)
Myogenic Tone
(% maximum)
Pressure (mmHg)

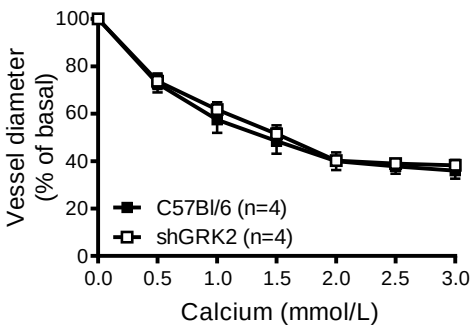
C)


C57Bl/6 (n=5)
shGRK2 (n=5)
PE-mediated constriction
(% of maximum)
Phenylephrine log [M]

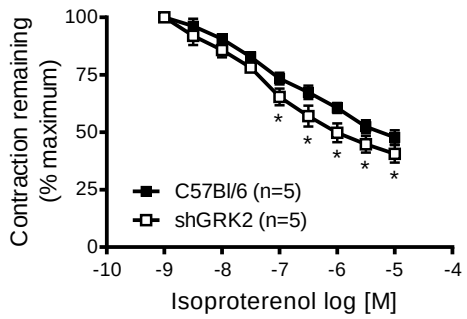
D)


C57Bl/6 (n=5)
shGRK2 (n=5)
KCl-mediated constriction
(% of maximum)
KCl (mmol/L)

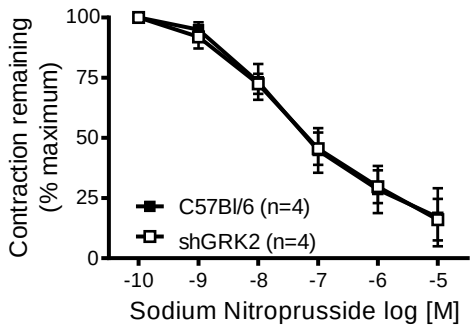
E)


C57Bl/6 (n=4)
shGRK2 (n=4)
Vessel diameter
(% of basal)
Calcium (mmol/L)

F)


C57Bl/6 (n=5)
shGRK2 (n=5)
Contraction remaining
(% maximum)
Isoproterenol log [M]

G)


C57Bl/6 (n=4)
shGRK2 (n=4)
Contraction remaining
(% maximum)
Sodium Nitroprusside log [M]

apparatus, we assessed KCl-mediated constriction in the presence of increasing concentrations of extracellular calcium. No significant differences in Ca^{2+} sensitivity were observed in mesenteric arteries obtained from shGRK2 and wild-type mice (Fig. 2.4E). To determine if GPCR-stimulated vasodilator pathways were altered in shGRK2 mice, we examined receptor-mediated vasodilation in response to isoproterenol in phenylephrine pre-constricted arteries. Isoproterenol-mediated vasodilation was significantly enhanced at 100 nM and greater doses (Fig. 2.4F, wild-type control EC_{50}: 107 ± 13 nM, n = 5 versus shGRK2 EC_{50}: 70 ± 7 nM, n = 5, $p < 0.05$). However, the non-receptor mediated vasodilation in response to sodium nitroprusside was not significantly different between shGRK2 and wild-type mouse arteries (Fig. 2.4G). In addition, the assessment of aortic arteries through H&E, Movat, and Masson staining has revealed that while control aortas from wild type mice have a normal tunica media characterized by a wavy look of the elastic lamina, GRK2 aortas are collapsed and have a straight elastic lamina (Fig. 2.5). On the other hand, no differences in collagen and elastin deposition were observed between shGRK2 and control aortas.

2.3.5 Heterotrimeric G Protein-mediated Signaling is Altered in shGRK2 VSMCs

Smooth muscle α-actin was expressed at relative constant levels over six different passages (Fig. 2.6A and 2.6B). To assess the effectiveness of shGRK2 knockdown with increasing passage *in vitro*, we examined GRK2 protein expression in passaged VSMCs and found that GRK2 knockdown was maintained in cultured shGRK2 VSMCs when compared to wild type VSMCs (Fig. 2.6C and 2.6D).

Figure 2.5: Histological analysis of aortas

Representative histological images of H&E, Movat staining, and Masson staining of aortic sections from shGRK2 and wild type C57BL/6 mice. Images were taken at 20x magnification and scale bar = 100 μm.

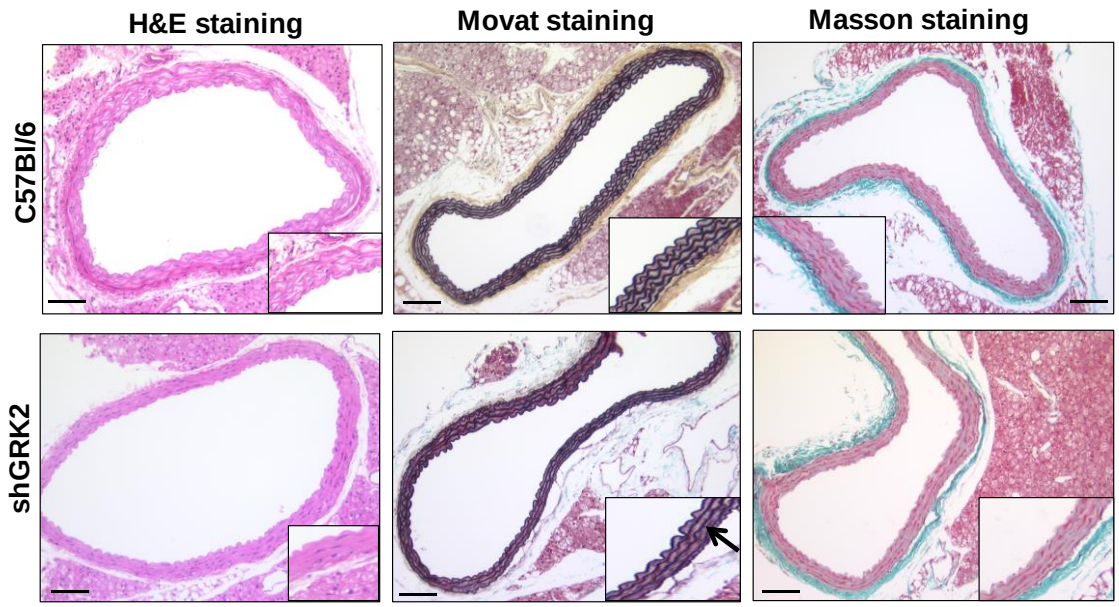
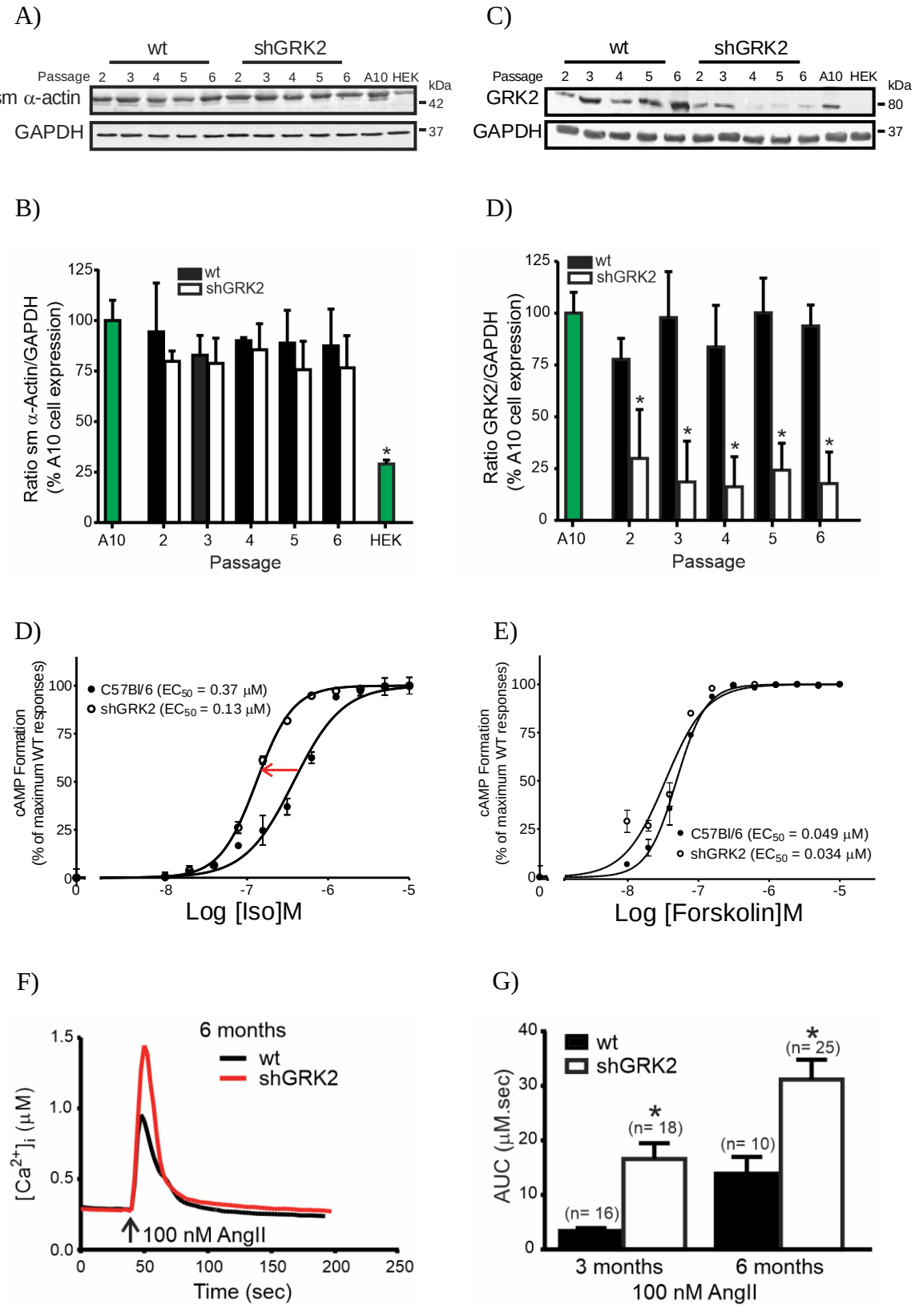

H&E staining
Movat staining
Masson staining
C57Bl/6
shGRK2

Figure 2.6: Assessment of GRK2 knockdown and heterotrimeric G protein mediated signaling in primary VSMCs

(A) Representative immunoblot for smooth muscle α-actin and GAPDH in VSMCs cultured from C57Bl/6 and shGRK2 mice. **(B)** Densitometric analysis of smooth muscle α-actin expression in C57Bl/6 and shGRK2 VSMCs over 2-6 passages in culture. A10 and HEK293 cells were used as positive and negative controls, respectively. Data is represented as the mean ± SD of 3 independent experiments. **(C)** Representative immunoblot for GRK2 and GAPDH in VSMCs cultured from C57Bl/6 and shGRK2 mice. **(D)** Densitometric analysis of GRK2 expression in wild-type and shGRK2 VSMCs over 2-6 passages in culture. A10 and HEK293 cells were used as positive and negative controls, respectively. Data is represented as the mean ± SD of 3 independent experiments. * Indicates statistically significant differences compared to time matched C57Bl/6 GRK2 expression ($p < 0.05$). Dose response curves for cAMP production in C57Bl/6 and shGRK2 VSMCs in response to treatment with increasing concentrations of **(E)** isoproterenol (Iso) and **(F)** forskolin. Data points are represented as the mean ± SEM of 4 independent experiments. **(G)** Representative Ca^{2+} tracers for changes in intracellular Ca^{2+} concentration in C57Bl/6 versus shGRK2 VSMCs cultures in response to treatment 100 nM AngII, **(H)** Graph shows the area under the curve (AUC) for integrated Ca^{2+} responses to agonist treatments. Data are represented as the mean ± SD of 6 independent experiments and the total number of cells analyzed is shown in the graph.


A)
wt
shGRK2
Passage 2 3 4 5 6 2 3 4 5 6 A10 HEK
kDa
sm α-actin
42
GAPDH
37
C)
wt
shGRK2
Passage 2 3 4 5 6 2 3 4 5 6 A10 HEK
kDa
GRK2
80
GAPDH
37
B)
Ratio sm α-Actin/GAPDH (% A10 cell expression)
wt
shGRK2
A10 2 3 4 5 6 HEK
Passage
D)
Ratio GRK2/GAPDH (% A10 cell expression)
wt
shGRK2
A10 2 3 4 5 6
Passage
D)
cAMP Formation (% of maximum WT responses)
C57Bl/6 (EC50 = 0.37 μM)
shGRK2 (EC50 = 0.13 μM)
Log [Iso]M
E)
cAMP Formation (% of maximum WT responses)
C57Bl/6 (EC50 = 0.049 μM)
shGRK2 (EC50 = 0.034 μM)
Log [Forskolin]M
F)
6 months
wt
shGRK2
[Ca2+]i (μM)
100 nM AngII
Time (sec)
G)
wt
shGRK2
AUC (μM.sec)
(n= 16)
(n= 18)
(n= 10)
(n= 25)
3 months
6 months
100 nM AngII

To determine whether alteration in GRK2 expression could exert an effect in $G\alpha_s$-mediated signaling, cyclic adenosine monophosphate (cAMP) accumulation in response to the β_2AR agonist isoproterenol was assessed. The EC_{50} for isoproterenol-stimulated cAMP accumulation in VSMCs prepared from 3 month-old mice was significantly reduced in shGRK2 VSMCs when compared to wild-type VSMCs (Fig. 2.6E).

In contrast, forskolin-stimulated cAMP formation was unchanged in shGRK2 versus control VSMCs (Fig. 2.6F). Thus, a loss of GRK2 expression appeared to lead to decreased desensitization of β_2-adrenergic receptor signaling. The release of Ca^{2+} from intracellular stores in response to treatment with 100 nM Ang II was assessed in VSMCs prepared from both 3 and 6 month-old shGRK2 and wild-type mice. We found that Ang II treatment elicited a significantly greater Ca^{2+} release from VSMCs cultured from 3 and 6 month-old shGRK2 mice, when compared to wild-type VSMCs (Fig. 2.6G and 2.6H). Taken together, these results indicated that GRK2 expression not only played a significant role in regulating GPCR desensitization in VSMCs, receptor responsiveness was significantly altered with age.

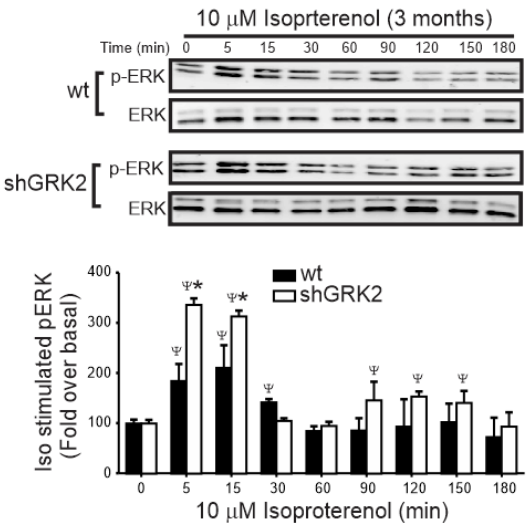
2.3.6 Effect of GRK2 Knockdown on ERK1/2 Phosphorylation

We compared isoproterenol-stimulated ERK1/2 phosphorylation with ERK1/2 phosphorylation responses elicited with the biased agonist carvedilol in VSMCs derived from 3 month-old shGRK2 and wild-type mice. Isoproterenol treatment (10 μM) of both wild-type and shGRK2 VSMCs elicited a rapid increase in ERK1/2 phosphorylation at 5 and 15 min of agonist treatment, but ERK1/2 phosphorylation responses were significantly greater in the VSMCs derived from shGRK2 mice (Fig. 2.7A). A second wave of ERK1/2 phosphorylation was also observed at 90-150 min of agonist stimulation in shGRK2, but

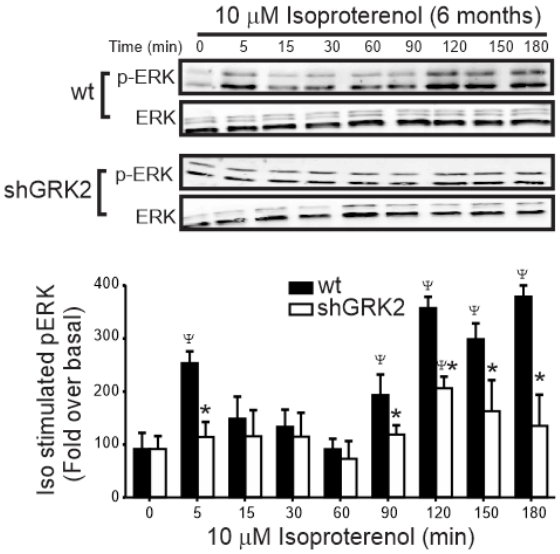
Figure 2.7: Assessment of $G\alpha_s$-mediated ERK1/2 signaling in primary VSMCs

Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VMSCs derived from 3 month-old wild-type (C57Bl/6) and shGRK2 mice in response to either **(A)** 10 μM isoproterenol or **(B)** 10 μM carvedilol. Data are represented as the mean ± SD of 4 independent experiments expressed as percentage of basal ERK1/2 phosphorylation normalized. Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VMSCs derived from 6 month-old wild-type (C57Bl/6) and shGRK2 mice in response to either **(C)** 10 μM isoproterenol or **(D)** 10 μM carvedilol. Total ERK1/2 was used as internal control. Data are represented as the mean ± SD of 4 independent experiments expressed as percentage of basal ERK1/2 phosphorylation. Ψ Indicates statistically significant differences compared to unstimulated C57Bl/6 control ($p< 0.05$). * Indicates statistically significant differences compared to time matched C57Bl/6 ERK1/2 ($p < 0.05$).

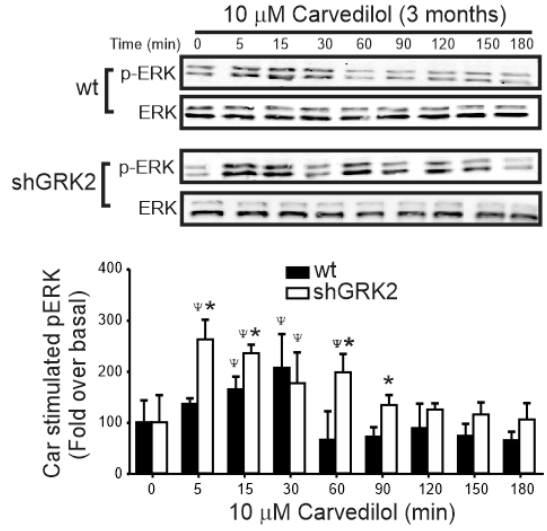
A)


10 μM Isoprterenol (3 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
Iso stimulated pERK (Fold over basal)
wt
shGRK2
10 μM Isoproterenol (min)

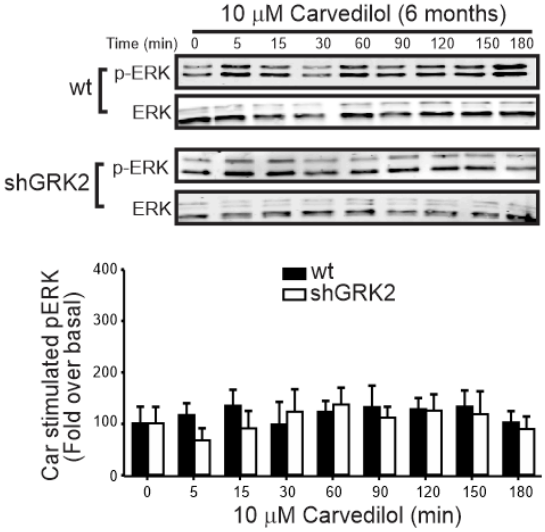
C)


10 μM Isoproterenol (6 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
Iso stimulated pERK (Fold over basal)
wt
shGRK2
10 μM Isoproterenol (min)

B)


10 μM Carvedilol (3 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
Car stimulated pERK (Fold over basal)
wt
shGRK2
10 μM Carvedilol (min)

D)


10 μM Carvedilol (6 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
Car stimulated pERK (Fold over basal)
wt
shGRK2
10 μM Carvedilol (min)

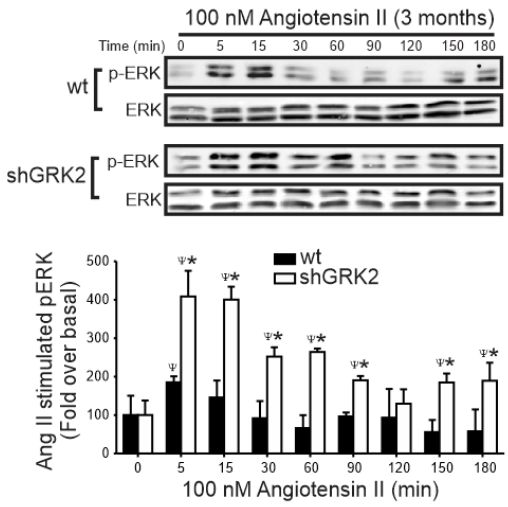
not wild-type VSMCs (Fig. 2.7A). Carvedilol treatment (10 μM) also resulted in an increase in ERK1/2 phosphorylation that was enhanced and prolonged in VSMCs derived from shGRK2 mice when compared to wild-type mice (Fig. 2.7B). In contrast, for VSMCs derived from 6 month old mice, the pattern of isoproterenol-stimulated ERK1/2 phosphorylation was significantly altered, with increases in ERK1/2 phosphorylation observed at latter time points of agonist treatment (Fig. 2.7C). Isoproterenol-stimulated ERK1/2 phosphorylation at later time points in VSMCs derived from 6 month-old shGRK2 mice was significantly impaired when compared with wild-type VSMC cultures (Fig. 2.7C). However, carvedilol did not elicit increased ERK1/2 phosphorylation in VSMCs derived from either 6 month- old shGRK2 or wild-type mice (Fig. 2.7D).

ERK1/2 phosphorylation in response to 100 nM Ang II treatment was also significantly increased and prolonged in shGRK2 versus wild-type VSMCs derived from 3 month-old mice (Fig. 2.8A). A similar response profile was observed in response to the treatment of 3 month-old shGRK2 and wild-type cultures with the biased angiotensin receptor agonist [Sar^1, Ile^4, Ile^8] Ang II (SII), except that a second later phase of ERK1/2 phosphorylation was observed in wild-type VSMCs that was not observed in response to Ang II treatment (Fig. 2.8B). Similar to what was observed for isoproterenol treatment of VSMCs derived from 6 month-old shGRK2 mice, Ang II-stimulated ERK1/2 phosphorylation was delayed (Fig. 2.8C). However, unlike what was observed for carvedilol, SII treatment mediated a modest biphasic increase in ERK1/2 phosphorylation in shGRK2 VSMCs that was not observed in wild-type VSMCs (Fig. 2.8D). Taken together, these data indicate that changes in GRK2 expression modulate ERK1/2 activity in response to both full and biased agonists and that these responses were altered with age.

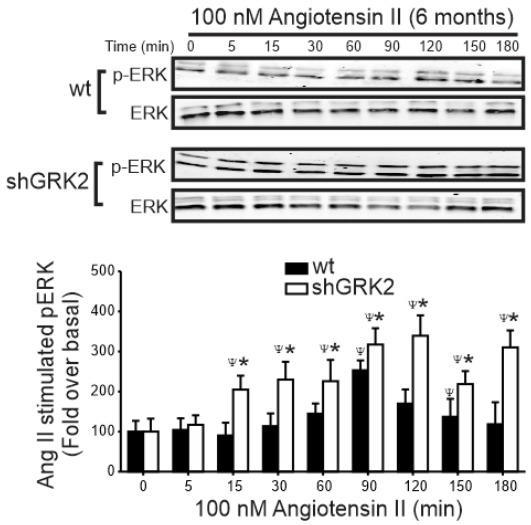
Figure 2.8*: Assessment of $G\alpha_q$-mediated ERK1/2 signaling in primary VSMCs*

Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VMSCs derived from 3 month-old wild-type (C57Bl/6) and shGRK2 mice in response to either **(A)** 100 nM AngII or **(B)** 100 μM SII. Data are represented as the mean ± SD of 4 independent experiments expressed as percentage of basal ERK1/2 phosphorylation. Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VMSCs derived from 6 month-old wild-type (C57Bl/6) and shGRK2 mice in response to either **(C)** 100 nM Ang II or **(D)** 100 μM SII. Total ERK1/2 was used as internal control. Data are represented as the mean ± SD of 4 independent experiments expressed as percentage of basal ERK1/2 phosphorylation. Ψ Indicates statistically significant differences compared to unstimulated C57Bl/6 control ($p< 0.05$). * Indicates statistically significant differences compared to time matched C57Bl/6 ERK1/2 ($p < 0.05$).

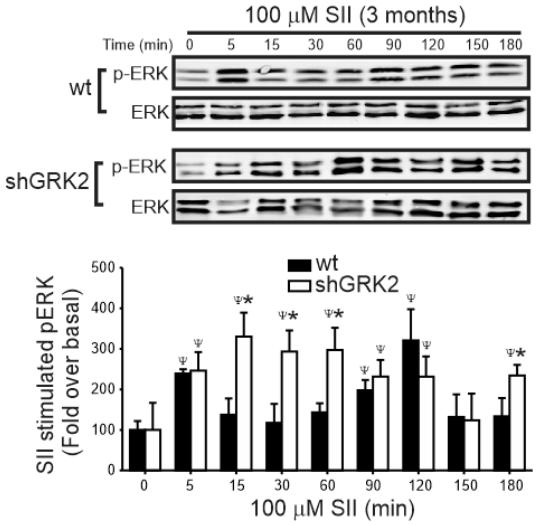
A)


100 nM Angiotensin II (3 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
wt
shGRK2
Ang II stimulated pERK (Fold over basal)
100 nM Angiotensin II (min)

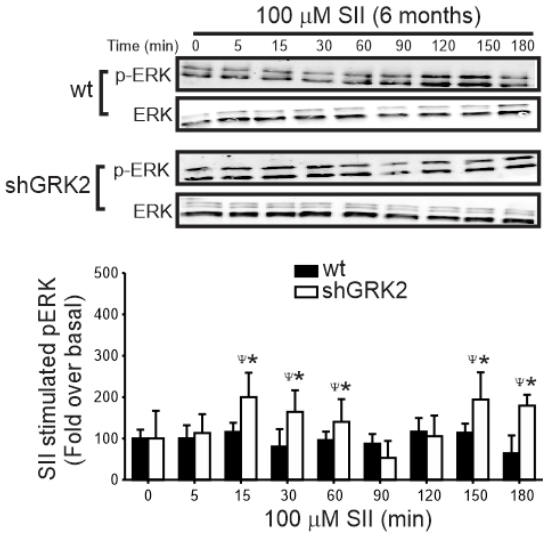
C)


100 nM Angiotensin II (6 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
wt
shGRK2
Ang II stimulated pERK (Fold over basal)
100 nM Angiotensin II (min)

B)


100 μM SII (3 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
wt
shGRK2
SII stimulated pERK (Fold over basal)
100 μM SII (min)

D)


100 μM SII (6 months)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
wt
shGRK2
SII stimulated pERK (Fold over basal)
100 μM SII (min)

2.3.7 Effect of GRK2 Knockdown on Akt Phosphorylation

β-Arrestins also couple GPCRs to the activation of Akt (Beaulieu *et al.*, 2005). Therefore, because GRK2 knockdown altered ERK1/2 phosphorylation responses following either β_2AR or AT1R activation, we assessed Akt signaling in VSMCs derived from 6 month-old wild-type and shGRK2 mice. We find that isoproterenol treatment of wild-type VSMCs resulted in a modest increase in Akt phosphorylation following 15 and 30 min agonist stimulation, whereas isoproterenol treatment of shGRK2 VSMCs results in a more robust, sustained and biphasic activation of Akt phosphorylation (Fig. 2.9A). In contrast, carvedilol did not induce Akt phosphorylation in wild-type VSMCs and provoked only a modest increase in Akt phosphorylation in shGRK2 VSMCs following 15, 30 and 180 min agonist treatment (Fig. 2.9B). Ang II and SII treatment of wild-type VSMCs did not significantly promote Akt phosphorylation (Fig. 2.9C and 2.9D). The stimulation of shGRK2 VSMCs with Ang II elicited the same robust, sustained and biphasic activation of Akt phosphorylation observed following isoproterenol treatment, and SII treatment induced a modest, but significant, increase in Akt phosphorylation (Fig. 2.9D). Taken together, these observations indicated that a loss of GRK2 expression favored increased Akt phosphorylation selectively in response to full receptor agonists. In addition, the phosphorylation status of both EKR1/2 and Akt signaling was assessed in response to stimulation with AngII and its biased agonist in VSMCs isolated from 6 week-old shGRK2 (Fig. 2.10). Thus, Ang II and SII treatment of both wild-type and shGRK2 VSMCs did not elicit an increase in ERK1/2 and Akt phosphorylation supporting the observation that the alteration of short and long term downstream signaling pathways is a consequence of the hypertensive phenotype.

Figure 2.9*:* Assessment of Akt signaling in primary VSMCs

Representative immunoblots and densitometric analysis for Akt phosphorylation at Ser 473 in primary VSMCs derived from 6 month-old wild-type (C57Bl/6) and shGRK2 mice in response to treatment with either **(A)** 10 μM isoproterenol, **(B)** 10 μM carvedilol, **(C)** 100 nM Ang II or **(D)** 100 μM SII. Total Akt was used as internal control. Data are represented as the mean ± SD of 3 independent experiments expressed as percentage of basal Akt phosphorylation. Ψ indicates statistically significant differences compared to unstimulated C57Bl/6 control ($p < 0.05$). * Indicates statistically significant differences compared to time matched C57Bl/6 Akt ($p < 0.05$).

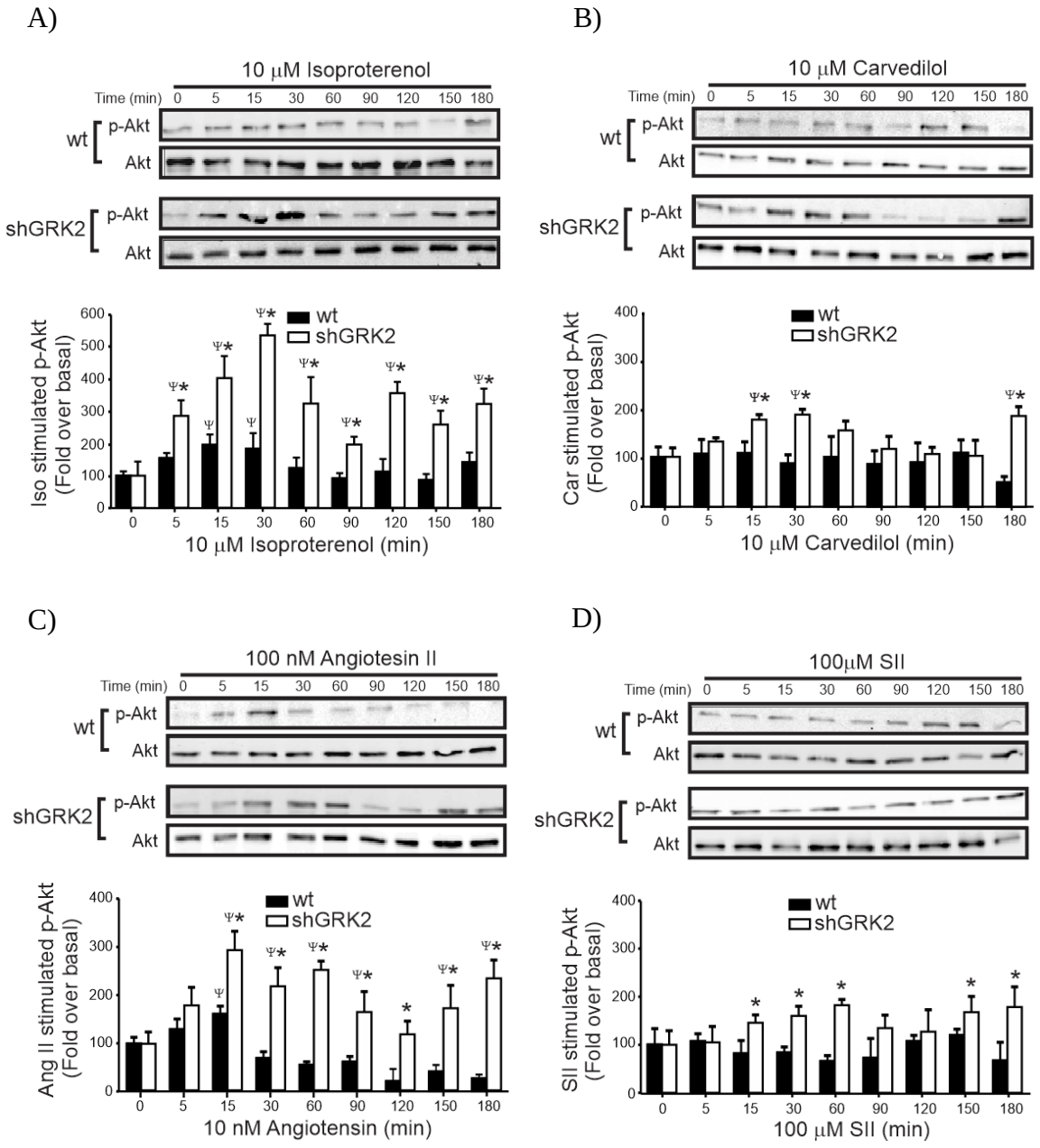
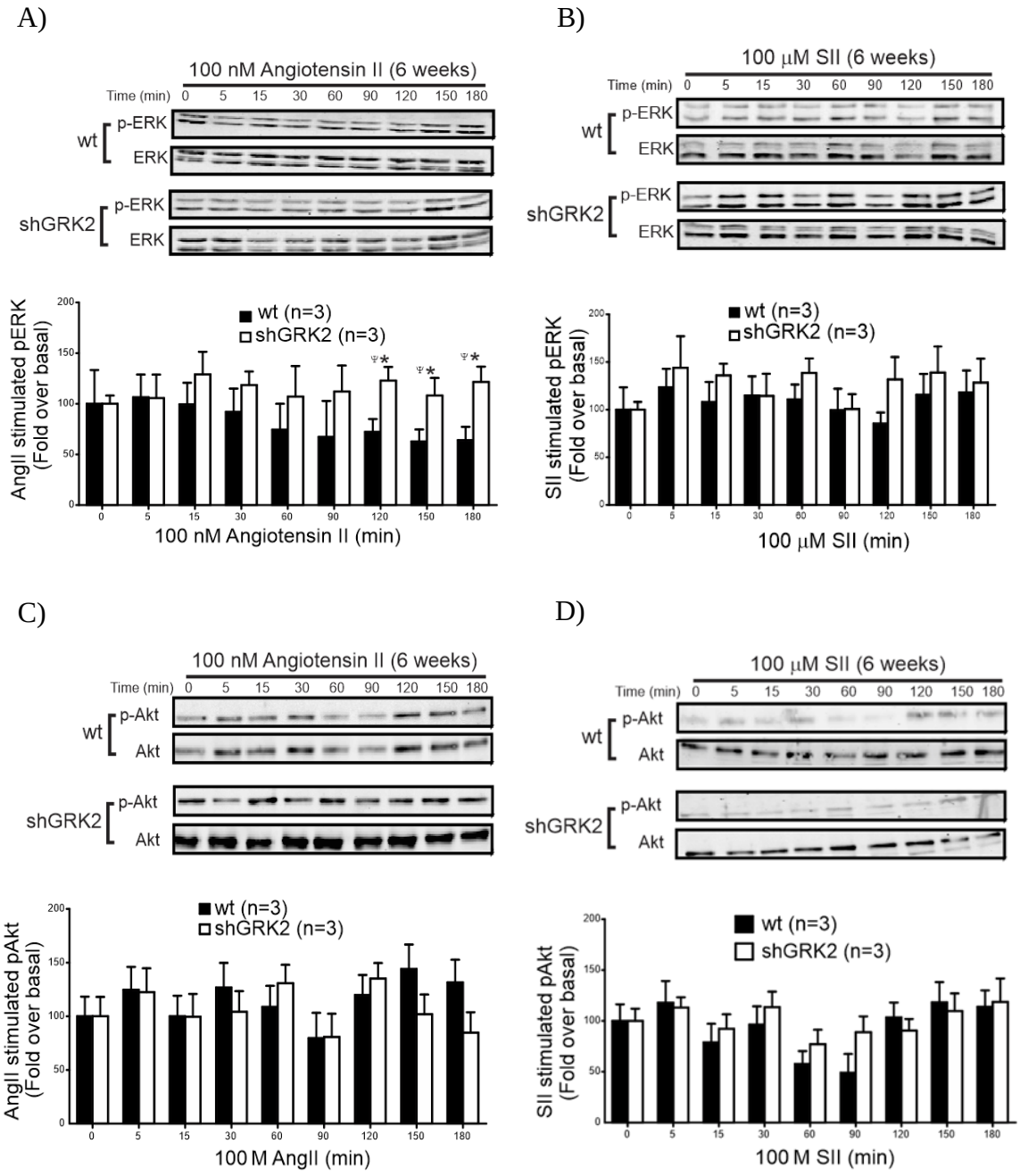

A)
10 μM Isoproterenol
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-Akt
Akt
shGRK2
p-Akt
Akt
Iso stimulated p-Akt (Fold over basal)
wt
shGRK2
10 μM Isoproterenol (min)
B)
10 μM Carvedilol
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-Akt
Akt
shGRK2
p-Akt
Akt
Car stimulated p-Akt (Fold over basal)
wt
shGRK2
10 μM Carvedilol (min)
C)
100 nM Angiotesin II
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-Akt
Akt
shGRK2
p-Akt
Akt
Ang II stimulated p-Akt (Fold over basal)
wt
shGRK2
10 nM Angiotensin (min)
D)
100μM SII
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-Akt
Akt
shGRK2
p-Akt
Akt
SII stimulated p-Akt (Fold over basal)
wt
shGRK2
100 μM SII (min)

Figure 2.10: Assessment ERK1/2 and Akt signaling in 6 week-old primary VSMCs

Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VMSCs derived from 6 week-old wild-type (C57Bl/6) and shGRK2 mice in response to either **(A)** 100 nM AngII or **(B)** 100 μM SII. Data are represented as the mean ± SD of 3 independent experiments expressed as percentage of basal ERK1/2 phosphorylation. Representative immunoblots and densitometric analysis showing time course for Akt phosphorylation with corresponding total Akt immunoblots from VMSCs derived from 6 week-old wild-type (C57Bl/6) and shGRK2 mice in response to either **(C)** 100 nM Ang II or **(D)** 100 μM SII. Data are represented as the mean ± SD of 3 independent experiments expressed as percentage of basal Akt phosphorylation. Ψ Indicates statistically significant differences compared to unstimulated C57Bl/6 control ($p< 0.05$). * Indicates statistically significant differences compared to time matched C57Bl/6 ($p < 0.05$).


A)
100 nM Angiotensin II (6 weeks)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
wt (n=3)
shGRK2 (n=3)
AngII stimulated pERK (Fold over basal)
100 nM Angiotensin II (min)
B)
100 μM SII (6 weeks)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-ERK
ERK
shGRK2
p-ERK
ERK
wt (n=3)
shGRK2 (n=3)
SII stimulated pERK (Fold over basal)
100 μM SII (min)
C)
100 nM Angiotensin II (6 weeks)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-Akt
Akt
shGRK2
p-Akt
Akt
wt (n=3)
shGRK2 (n=3)
AngII stimulated pAkt (Fold over basal)
100 M AngII (min)
D)
100 μM SII (6 weeks)
Time (min) 0 5 15 30 60 90 120 150 180
wt
p-Akt
Akt
shGRK2
p-Akt
Akt
wt (n=3)
shGRK2 (n=3)
SII stimulated pAkt (Fold over basal)
100 M SII (min)

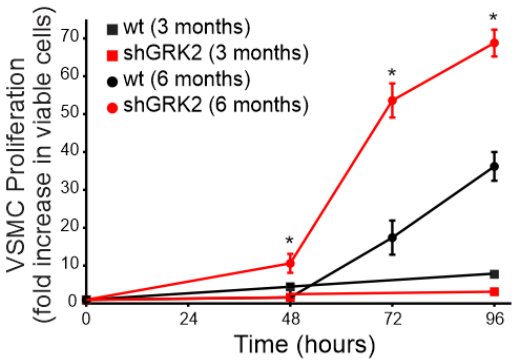
2.3.8 Effect of GRK2 Knockdown on VSMC Proliferation

We found that wild-type and shGRK2 VSMCs derived from 3 month old mice exhibited a limited proliferation rate (Fig. 2.11A). In contrast, VSMCs derived from 6 month-old wild-type and shGRK2 mice exhibited a more robust proliferative phenotype than VSMCs derived from 3 month old mice, with shGRK2 VSMCs exhibiting a significantly greater proliferation rate that wild-type VSMCs (Fig. 2.11A). Agonist treatment with either 10 μM isoproterenol, 10 μM carvedilol, 100 nM Ang II or 100 μM SII elicited a significant increase in 5-bromo-2-deoxyuridine (BrdU) incorporation in VSMCs derived from 3 month-old wild-type and shGRK2 mice when compared with serum free medium (Fig. 2.11B). For cells derived from 6 month-old mice, neither isoproterenol, carvedilol, Ang II nor SII treatment increased BrdU incorporation into wild-type VSMCs, whereas only Ang II and SII treatment increased BrdU incorporation into shGRK2 VSMCs (Fig. 2.11C). Treatment of shGRK2 VSMCs with either an Akt inhibitor (Akti-1/2), phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) or ERK1/2 inhibitor (U0126) inhibited Ang II-stimulated BrdU incorporation (Fig. 2.11D). These data suggested that the activation of AT_1 receptor in shGRK2 mice selectively activates cell proliferation of older VSMCs in a PI3K/Akt/ERK-dependent manner.

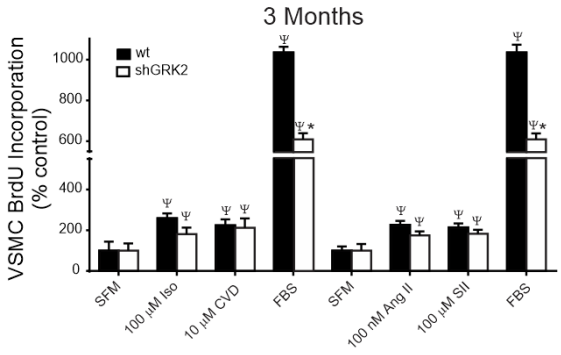
Figure 2.11*: Assessment of proliferation responses for primary VSMCs*

(A) Growth curves over 96-hour course time of primary VSMCs cultured from either 3 or 6 month-old wild-type (C57Bl/6) and shGRK2 mice. Data are represented as the mean ± SD (n = 4). **(B)** Proliferation responses of VSMCs cultured from 3 month-old wild-type (C57Bl/6) and shGRK2 treated for 24h with serum free medium (SFM), 10 μM isoproterenol, 10 μM carvedilol, 100 nM Ang II or 100 μM [Sar^1, Ile^4, Ile^8] Ang II (SII) as measured by BrdU incorporation in newly synthesized cellular DNA. Data are represented as the mean ± SD of 4 independent experiments. **(C)** Proliferation responses of VSMCs from 6 month old wild-type (C57Bl/6) and shGRK2 treated for 24h with SFM, 10 μM isoproterenol, 10 μM carvedilol, 100 nM Ang II or 100 μM [Sar^1, Ile^4, Ile^8] Ang II (SII) as measured by BrdU incorporation in newly synthesized cellular DNA. Data are represented as the mean ± SD of 4 independent experiments. **(D)** Cell proliferation responses in VSMCs cultured from 6 month-old shGRK2 treated with SFM or 100 nM Ang II for 24h in the presence or absence of either 10 μM Akti-1/2, 50 μM LY294002, or 1 μM U0126 inhibitors. Data are represented as the mean ± SD of 4 independent experiments. *Indicates statistically significant differences compared to unstimulated control ($p < 0.05$).

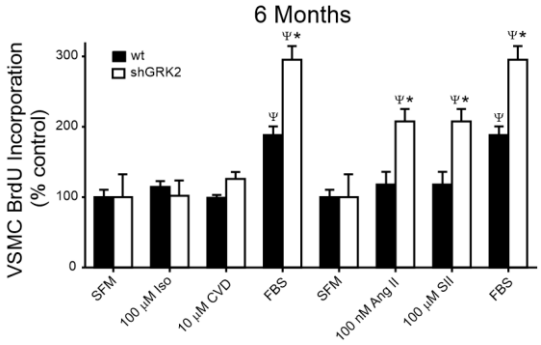
A)


wt (3 months)
shGRK2 (3 months)
wt (6 months)
shGRK2 (6 months)
VSMC Proliferation
(fold increase in viable cells)
Time (hours)
0
24
48
72
96

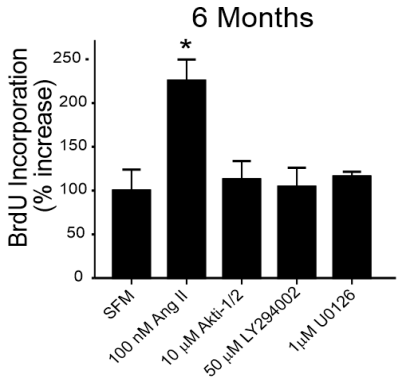
B)


3 Months
wt
shGRK2
VSMC BrdU Incorporation
(% control)
SFM
100 μM Iso
10 μM CVD
FBS
SFM
100 nM Ang II
100 μM SII
FBS

C)


6 Months
wt
shGRK2
VSMC BrdU Incorporation
(% control)
SFM
100 μM Iso
10 μM CVD
FBS
SFM
100 nM Ang II
100 μM SII
FBS

D)


6 Months
BrdU Incorporation
(% increase)
SFM
100 nM Ang II
10 μM Akti-1/2
50 μM LY294002
1μM U0126

2.3.9 Effect of GRK2 Knockdown on VSMC Migration

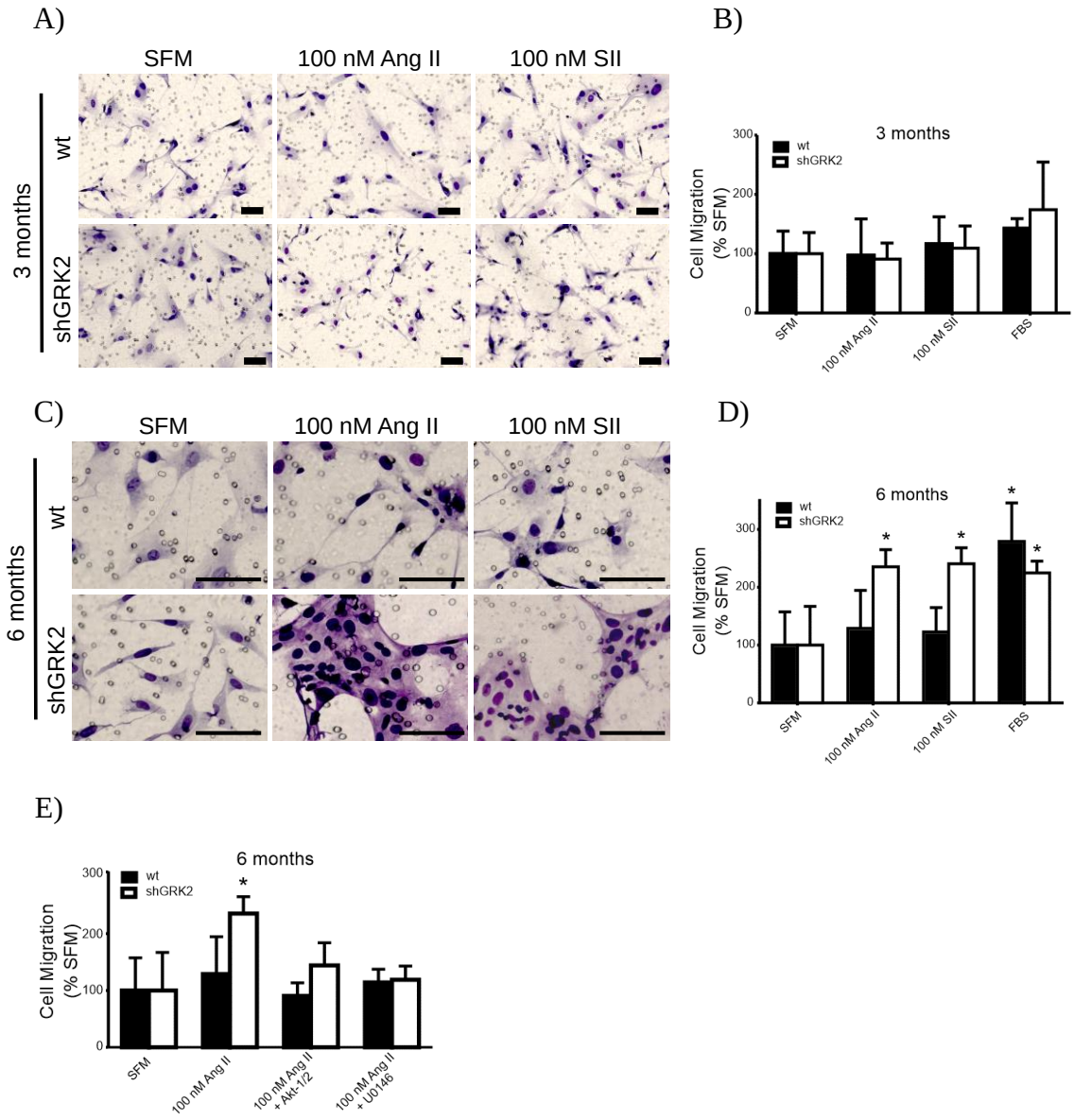
Migration assays for wild-type and shGRK2 VSMCs derived from 3 month old mice revealed that neither 100 nM Ang II or 100 μM SII stimulated cellular migration when compared to serum-free medium (Fig. 2.12A and 2.12B). However, the treatment of VSMCs derived from 6 month-old wild-type and shGRK2 mice with either Ang II or SII significantly increased shGRK2, but not wild-type, VSMCs migration as compared to serum-free medium (Fig. 2.12C and 2.12D). The treatment of shGRK2 VSMCs with either Akt or ERK1/2 inhibitors prevented Ang II-induced shGRK2 VSMCs migration (Fig. 2.12E). Thus, GRK2 knockdown increased VSMCs migration in response to $G\alpha_{q/11}$-coupled GPCR activation.

2.4 Discussion

GRK2 contributes to the desensitization of GPCR signaling and generalized increases in GRK2 expression are linked to the development of essential hypertension in both humans and experimental animal models (Gros *et al.*, 1997; Gros *et al.*, 1999; Gros *et al.*, 2000; Eckhart *et al.*, 2002; Harris *et al.*, 2007; Cohn *et al.*, 2008; Izzo *et al.*, 2008; Cohn *et al.*, 2009; Morris *et al.*, 2010; Napolitano *et al.*, 2012; Avendano *et al.*, 2014). To further understand the role of the balance between GRK2 expression and vascular reactivity, we developed a shGRK2 transgenic mouse. The shGRK2 transgenic mice are viable, show indications of intrauterine growth retardation and become spontaneously hypertensive at 8-12 weeks following birth. GRK2 knockdown both increases vasodilator and vasoconstrictor responses to GPCR activation in intact tissue and VSMC cultures, but increase in vasoconstrictor mechanisms appear to dominate the physiological phenotype.

Figure 2.12*:* Assessment of migration responses for primary VSMCs

(A) Representative images and **(B)** quantification of the migration of VSMCs derived from wild-type (C57Bl/6) and 3 month- old shGRK2 mice treated for 24h SFM, 100 nM Ang II, or 100 μM [Sar^1, Ile^4, Ile^8] Ang II (SII). Bar graph data represents the mean ± SD of 4 independent experiments. **(C)** Representative images and **(D)** quantification of the migration of VSMCs derived from wild-type (C57Bl/6) and 6 month- old shGRK2 mice treated for 24h with SFM, 100 nM Ang II, or 100 μM [Sar^1, Ile^4, Ile^8] Ang II (SII). Bar graph data represents the mean ± SD of 4 independent experiments. **(E)** Quantification of cell migration responses of VSMCs cultured from 6 month-old shGRK2 treated with SFM or 100 nM Ang II for 24h in the presence or absence of either 10 μM Akti-1/2, or 1 μM U0126 inhibitors. Data represents the mean ± SD of 4 independent experiments. In **(A, C)** the microphotographs were taken at 40x magnification and scale bar = 50 μm. *Indicates statistically significant differences compared to unstimulated control ($p < 0.05$).


A)
SFM
100 nM Ang II
100 nM SII
3 months
wt
shGRK2
B)
3 months
wt
shGRK2
Cell Migration (% SFM)
300
200
100
0
SFM
100 nM Ang II
100 nM SII
FBS
C)
SFM
100 nM Ang II
100 nM SII
6 months
wt
shGRK2
D)
6 months
wt
shGRK2
Cell Migration (% SFM)
300
200
100
0
*
SFM
100 nM Ang II
100 nM SII
FBS
E)
6 months
wt
shGRK2
Cell Migration (% SFM)
300
200
100
0
*
SFM
100 nM Ang II
100 nM Ang II + Akt-1/2
100 nM Ang II + U0146

VSMCs isolated from older hypertensive mice also show alterations in ERK1/2 and Akt signaling, which are linked to altered cellular proliferation and migration responses to $G\alpha_{q/11}$-coupled GPCR activation.

GRK2/3 are unique with respect to other GRK family members in that they mediate the GRK2 RGS homology domain-dependent phosphorylation-independent desensitization of only $G\alpha_{q/11}$-coupled GPCRs (Ferguson, 2007). Consequently, significant reductions in GRK2 protein expression may preferentially result in enhanced signaling via $G\alpha_{q/11}$-coupled GPCRs leading to increased vasoconstrictor activity and hypertension. Thus, the current results are not incongruent with previous studies linking increased GRK2 expression to hypertension, as reduced GRK2 expression, given the importance of GRK2-phosphorylation-independent desensitization in the regulation of $G\alpha_{q/11}$-coupled GPCR signaling.

GRK2 expression is associated with both enhanced βAR desensitization in lymphocytes and impaired vascular reactivity in hypertensive humans and increased GRK2 expression in umbilical arteries is correlated with gestational hypertension (Gros *et al.*, 1997; Gros *et al.*, 1999; Napolitano *et al.*, 2012). VSMC-specific overexpression of GRK2 enhances βAR desensitization, attenuates βAR-mediated vasodilation, increases medial VSM thickness and results in cardiac hypertrophy (Eckhart *et al.*, 2002). The reduction of GRK2 expression in hemizygous GRK2 mice also partially protects against Ang II-mediated hypertension and vascular remodeling via a mechanism involving the preservation of nitric oxide availability, but does not affect resting blood pressure (Avendano *et al.*, 2014). We observed an age-dependent onset of hypertension in shGRK2 knockdown mice with a full onset of hypertension observed at 12 weeks of age, which is

maintained throughout the lifespan of the animals. The increases in blood pressure are not confounded by stress and/or anxiety as elevated blood pressure is also observed in lightly anesthetized mice. We also find that vasodilatation in response to βAR stimulation is enhanced in the shGRK2 mice, but consistent with the hypertensive phenotype vasoconstriction in response to PE is also increased and may predominate physiologically. In addition, myogenic response was increased in shGRK2 mouse mesenteric arteries and this has been linked to $G\alpha_{q/11}$-mediated pathways. These observations suggest that extensive loss of GRK2 expression favors an increase in vasoconstriction associated with increases in peripheral resistance likely due to reduced $G\alpha_{q/11}$-coupled receptor desensitization. This is consistent with the observation that inhibition of VSMC-GRK2 by either the overexpression of the GRK2-ct or VSMC-specific ablation of GRK2 protein expression increases α_{1D}AR-stimulated vasoconstriction. Consequently, it appears that either increased or decreased GRK2 expression can lead to a dysregulation in the balance between vasoconstrictor- and vasodilator-regulated vessel tone. $G\alpha_S$-coupled GPCRs that mediate vasodilatation are regulated solely by GRK2-mediated phosphorylation, whereas $G\alpha_{q/11}$-coupled receptors are regulated by both GRK2 phosphorylation-dependent and -independent mechanisms (Ferguson, 2007). Therefore, $G\alpha_S$-coupled GPCRs may be more sensitive to small increases in GRK2 expression due to the loss of phosphorylation-dependent desensitization, whereas a loss of GRK2 expression increases both $G\alpha_S$- and $G\alpha_{q/11}$-GPCR signaling. Thus, the shGRK2 hypertensive phenotype may be the consequence of extent of GRK2 suppression achieved in this model, due to the reduction of the $G\alpha_{q/11}$-selective uncoupling of $G\alpha_{q/11}$-coupled GPCRs by the, GRK2 RGS homology domain, which is not observed for $G\alpha_s$-coupled GPCRs (Ferguson, 2007). Consequently,

therapeutic strategies that target GRK2 activity, as opposed inhibiting receptor interactions, may be more effective for the treatment of hypertension.

Consistent with the observation that vasodilatation in response to βAR stimulation is increased in the shGRK2 mice, isoproterenol-stimulated cAMP formation is enhanced shGRK2 VSMCs when compared to wild-type mice indicating that βAR desensitization is reduced. We also observed that Ang II-stimulated Ca^{2+} release is increased in VMSCs cultured from shGRK2 mice suggesting that there is reduced GRK2-mediated desensitization of $G\alpha_{q/11}$ signaling. This is similar to what was previously observed for endothelin receptor signaling in VSMCs transfected with a GRK2-K220R/D110A mutant that does not mediate receptor desensitization (Morris *et al.*, 2010). Thus, in general, VSMCs responses recapitulate responses observed in intact tissue.

The ERK1/2 pathway is coupled to enhanced contractile responses and increased VSMC proliferation and the inhibition of ERK1/2 activity significantly reduces smooth muscle cell growth (Mii *et al.*, 1996; Dessy *et al.*, 1998; Touyz *et al.*, 2002; Kim *et al.*, 2005). We also observed alterations in ERK1/2 and Akt signaling. Specifically, we find that reduced GRK2 expression results in a significant increase in the extent and duration of Ang II- stimulated ERK1/2 and Akt phosphorylation in VSMC cultures derived from 6 month-old shGRK2 mice. This is in alignment with previous observations that GRK2 expression exerts a negative effect on β-arrestin signaling by competing with GRK5 and GRK6 for receptor binding (Zidar *et al.*, 2009). The time course for ERK1/2 phosphorylation in response to Ang II shifts with age with more effective activation of ERK1/2 phosphorylation at early time points in 3 month cultures and a more prolonged and delayed activation in 6 month cultures. This supports the concept that β-arrestin-

mediates the prolonged activation of ERK1/2 (Ahn *et al.*, 2004). Carvedilol and SII have been shown to activate ERK1/2 phosphorylation solely via the β-arrestin-dependent mechanism (Wei *et al.*, 2003; Wisler *et al.*, 2007). We found that both carvedilol and SII treatment results in increased ERK1/2 phosphorylation in VSMCs derived from 3 month-old wild-type and shGRK2 mice, but ERK1/2 phosphorylation is selectively increased in shGRK2 VSMCs. However, the treatment of VSMCs derived from 6 month-old wild-type mice with either carvedilol or SII does not result in either ERK1/2 or Akt phosphortylation, whereas in shGRK2 cultures SII, but not carvedilol stimulates ERK1/2 phosphorylation. In contrast, both carvedilol and SII stimulation elicit Akt phosphorylation in VSMCS dervied from 6 month old shGRK2 mice. In addition, we observed increases in ERK1/2, but not Akt signaling in response to Ang II in VSMCs derived from 6 week-old shGRK2 mice prior to the development of hypertension. Thus, it appears that alterations in both ERK1/2 and Akt are age-dependent and progress with the generation of the hypertensive phenotype, as pre-hypertensive mice do not exhibit a generalized alteration in both ERK1/2 and Akt activation in response to both Ang II and SII. Taken together, these observations indicate that GRK2 expression antagonizes ERK1/2 and Akt phosphorylation in VSMCs and the pattern of responsiveness to drug treatment changes with the development of hypertension. This suggests that the responsiveness to biased agonists may be reduced with age and might potentially limit their window of therapeutic effectiveness.

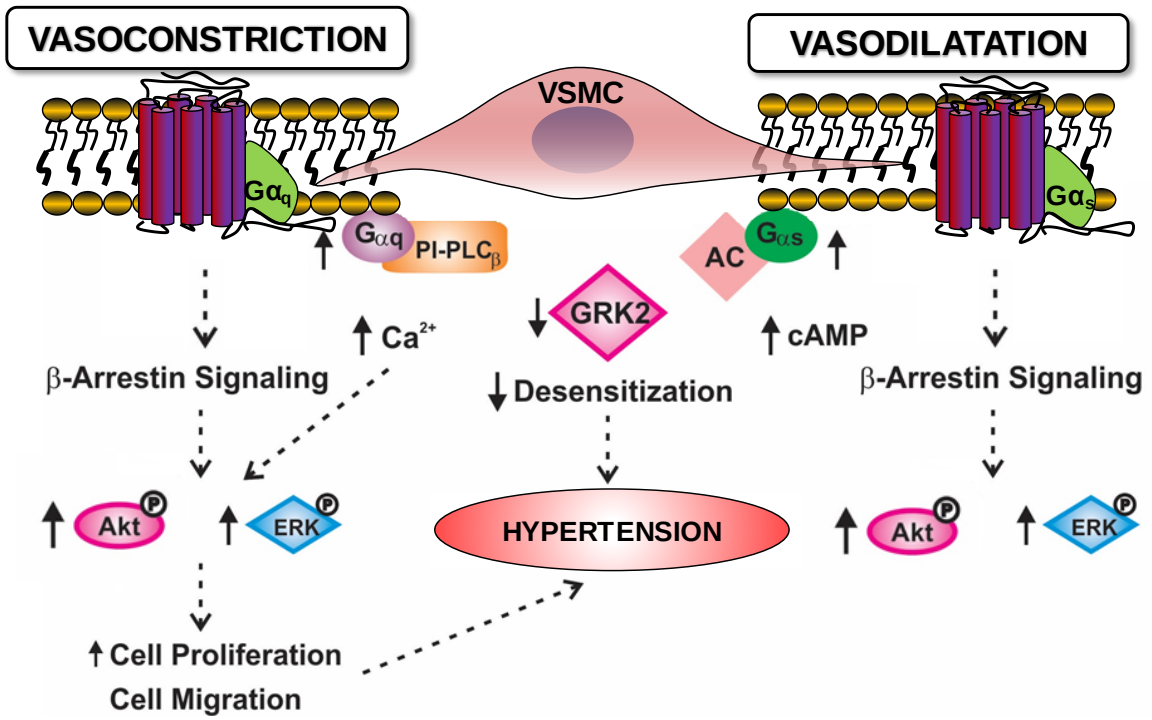
GRK2 plays an inhibitory role in cell cycle progression and GRK2 overexpression reduces smooth muscle, thyroid cancer and hepato-carcinoma cell growth and that GRK2 and β-arrestin2 regulate cell migration (Peppel *et al.*, 2000; Kim *et al.*, 2008; Metaye *et al.*, 2008; Lafarga *et al.*, 2012; Liu *et al.*, 2012; Wei *et al.*, 2012). The proliferation and

migration of VSMCs derived from older shGRK2 mice is significantly increased in response to Ang II stimulation via an ERK1/2- and Akt-dependent mechanism. β-Arrestin2 deletion reduces both VSMCs proliferation and migration and blocking GRK2 activity increases neutrophil migration (Kim *et al.*, 2008; Liu *et al.*, 2012). Thus, increased β-arrestin signaling in the absence of GRK2 expression promotes VSMCs proliferation and migration, which may contribute to the age-dependent onset of hypertension in shGRK2 mice. These increased proliferative and migratory responses are only observed following AT1R activation of VSMCs derived from shGRK2 mice. Thus, the context of the activation of the ERK/Akt pathway is likely also important in that $G\alpha_{q/11}$, but not $G\alpha_s$, receptor activation leads to proliferation and may require a concomitant increase in Ca^{2+} release from intracellular stores.

In summary, global knockdown of GRK2 expression results in a mouse that spontaneously develops hypertension, due to alterations in the balance between mechanisms regulating vasodilatation and vasoconstriction (Fig. 2.13). The loss of GRK2 expression not only results in increases in GPCR responsiveness to both vasodilators and constrictors, associated with reduced receptor desensitization, VSMC proliferation and migration are also increased as the consequence of what appears to be β-arrestin-mediated signaling. This highlights the complexity of the balance between G protein-dependent and -independent signaling pathways engaged by GPCRs.

Figure 2.13: Schematic diagram of the proposed role of GRK2-targeted knockdown expression in the mechanism of hypertension

Vascular smooth muscle cells cultured from shGRK2 knockdown mice show increases in GPCR-mediated $G\alpha_s$ and $G\alpha_{q/11}$ signaling, as the consequence of reduced GRK2-mediated desensitization. Global knockdown of GRK2 expression shifts the balance of GPCR-regulated vasodilator and vasoconstrictor mechanisms in the favor of $G\alpha_{q/11}$ that at least in part is responsible for the hypertensive phenotype observed in shGRK2 transgenic mice. In addition, agonists and biased agonists exhibited age-dependent alterations in ERK1/2 and Akt signaling pathways that are linked to altered cellular proliferation and migration responses to $G\alpha_{q/11}$-coupled GPCR activation and thereby could be responsible for the increase in peripheral resistance associated with the hypertensive phenotype.


VASOCONSTRICTION
VASODILATATION
VSMC
Gαq
Gαq
PI-PLCβ
AC
Gαs
Gαs
GRK2
Ca2+
cAMP
β-Arrestin Signaling
Desensitization
β-Arrestin Signaling
Akt
ERK
HYPERTENSION
Akt
ERK
Cell Proliferation
Cell Migration

These observations indicate that, while partial inhibition of GRK2 may be of therapeutic benefit, total inhibition of GRK2 expression may lead to alterations in GPCR signaling that will lead to the development of hypertension or other pathological conditions.

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CHAPTER 3:

INHIBITION OF GRK2 EXPRESSION PROMOTES KIDNEY INJURY AND DYSREGULATION OF RENAL-MEDIATED MECHANISMS OF BLOOD PRESSURE

3.1 Introduction

Blood pressure homeostasis is controlled through the interaction of multiple and complex organ systems. Kidneys play an essential permissive role in the elevation of blood pressure and dysregulation of the renal excretion function is a prerequisite for hypertension (Guyton, 1991). The strong correlation between genetic disorders affecting blood pressure homeostasis and those controlling the salt and water reabsorption at the nephron level (Lifton *et al.*, 2001) can be regarded as an unquestionable validation of the important modulatory role played by renal excretory function on systemic blood pressure.

The defective renal sodium excretion represents one of the common pathways that is responsible for the onset of the elevated blood pressure, regardless if induced on a vascular, neural or inflammatory basis. Along these lines, Aperia *et al.* (1971) proposed that elevations of the perfusion pressure in renal artery lead to "pressure natriuresis", a phenomenon characterized by rapid escalations of sodium and water excreted by the kidney in an attempt to restore normal values of the systemic blood pressure (Guyton *et al.*, 1972).

The two main regulatory pathways with a significant effect on sodium balance are the dopaminergic and renin-angiotensin system (RAS). As expected, these two systems are characterized by an antagonistic functionality that is modulated by a multitude of intracellular pathways with role in sodium and water homeostasis.

Any dysregulations and/or sustained activations of the RAS could translate into hypertension and end-organ damage (Gavras *et al.*, 1971; Brunner *et al.*, 1972; Brunner, 2001), primarily through the activity of AT_1 receptors (Lee *et al.*, 1988; Mazzolai *et al.*, 2000). These receptors are expressed in key tissues (kidney, vasculature, central and

sympathetic nervous systems) with a strong impact on the regulation of the peripheral vascular resistance and sodium balance. However, the relative contribution brought by each of these sites to the overall pathogenesis of the hypertension is difficult to distinguish. Prior studies have suggested that AT_1 receptors expressed by kidney and systemic tissues are equal and non-redundant contributors to the preservation of the normal blood pressure in the basal state, typically by protecting fluid body volumes to prevent circulatory collapse (Crowley *et al.*, 2005). Other studies have suggested that renal AT_1 receptors have in fact a dominant role on sodium retention and hence blood pressure elevation (Crowley *et al.*, 2006). The kidney cross-transplantation approach with AT_1 receptors eliminated from kidney and/or systemic tissues , suggest that – the absence of renal AT_1 receptors alone was sufficient to protect from AngII-dependent hypertension and cardiac hypertrophy(Crowley *et al.*, 2006).

Among the seven GRKs, GRK2 is unique with respect to other GRK family members in that it mediates the RGS homology domain-independent desensitization of only $G\alpha_{q/11}$-coupled GPCRs. Indeed, global knockdown of GRK2 expression results in mice that show enhanced signaling via $G\alpha_{q/11}$-coupled GPCRs leading to increased vasoconstrictor activity and hypertension (Tutunea-Fatan *et al.*, 2015). GRK2 contributes to the desensitization of GPCR signaling and it appears that either increased or decreased in GRK2 expression can lead to a dysregulation in the balance between vasoconstrictor- and vasodilator-regulated vessel tone. On the other hand, GRK4 is the only GRK subtype capable to phosphorylate unstimulated GPCRs due to its capacity to bind to inactive $G\alpha_s$ and $G\beta$ subunits (Jose *et al.*, 2010). GRK4 gene variants were shown to phosphorylate, desensitize, and internalize dopamine type 1(D_1R) and 3 (D_3R) receptors by preventing

their recycling to the plasma membrane. In addition, GRK4 gene variants were found to be responsible for stimulation of the AT_1 receptor expression (Felder *et al.*, 2002). The simultaneous elevated levels of GRK4 and AT_1R in kidney translate in higher blood pressure since their concurrent selective renal silencing was associated with increased sodium excretion and lower blood pressure (Yatabe *et al.*, 2008; Gurevich *et al.*, 2012).

In the present chapter, we have investigated the effects of reduced GRK2 expression on renal function and thereby the contribution of the kidney to the development of hypertension. The extensive loss of GRK2 expression leads to reduced desensitization of AT_1 receptors – expressed not only by vascular smooth muscle cells but also by kidney tissue – with a strong impact on the regulation of both peripheral vascular resistance and sodium balance. Thereby, these intricate effects seem to complement each other in the onset of hypertension. Our results indicate that as the blood pressure increases, the expression of renal RAS components increases correspondingly and associates with a decrease in urinary sodium excretion and thereby could be responsible – at least in part – for the hypertensive phenotype. The chronic activation of RAS not only leads to defective renal-sodium excretion but also potentiates renal injury by inducing alteration in glomerular filtration rates and progression of renal fibrosis. GRK2-mediated hypertension was also associated with an enhanced in mRNA expression level of different GRKs subtypes (GRK3, GRK4, and GRK5). Moreover, the pattern of their expression has been noticed to increase with the generation of the hypertensive phenotype. While GRK5 elevation endeavors a more compensatory role in an attempt to lower blood pressure, GRK3 and GRK4 upregulation has a more pro-hypertensive action. The effect of GRK4 activation on the dopaminergic-mediated natriuresis might potentiate the alteration of

sodium excretion and consequently contribute the blood pressure elevation.

3.2 Experimental Procedure

3.2.1 Morphometric Characterization of shGRK2 Kidney

The GRK2 hemizygous (GRK2 +/-) mice were generated on the C57Bl/6 genetic background as previously described (Tutunea-Fatan *et al.*, 2015). Kidneys (left and right) were collected, stripped of peritoneal fat and connective tissue and weights were recorded. Quantification of kidney mass relative to both body weight and tibia length was performed in a paired manner. All experiments involving animals were approved by the Animal Use Subcommittee of Western University, according to the guidelines of the Canadian Council on Animal Care.

3.2.2 Computed Tomography Analysis

Three age-based groups of 3, 6, and 12 month-old GRK2 (+/-) mice and control C57Bl/6 were used in this study. Mice were fed a regular diet and housed on a 12:12 hour light-dark cycle. Each mouse was anesthetized for the entire duration of the procedure (2 min) by exposure to 2.5% isoflurane-oxygen gas. A GE eXplore CT 120 scanner was used for 3D-images acquisition. Data were analyzed with MicroCT Visualization & Analysis software (MicroView) (Robarts Research Institute). All procedures were performed with the approval of the Western University Animal Ethics Committee.

3.2.3 Blood and Urine Analysis

The whole blood was collected into BD Microtainer Serum Separator Tubes (Becton-Dickinson, Franklin, NJ, USA) through cardiac puncture of mice under terminal anesthesia. Then, blood was allowed to clot at room temperature for one hour. Following

centrifugation at 1200 x g for 10 minutes, the resulting supernatant was collected, and stored immediately at -80 C for further biochemical analysis. Urine samples from conscious, restrained mice were collected in 1.5 ml microtubes and subjected to Chemistrip urinalysis test (Roche Diagnostics, Laval, Quebec, Canada) to detect the presence of glucose, protein, and blood. The analysis of biochemical constituents of blood and urine samples was performed at Mount Sinai Hospital Laboratory (Toronto, Ontario, Canada).

3.2.4 Measurement of Inulin Clearance

The glomerular filtration rate (GFR) was measured in conscious mice by assessing plasma kinetics of fluorescein isothiocyanate (FITC) labeled inulin (Sigma Aldrich, Saint Louis, Mo, USA). In short, FITC-inulin solution (2µl/g) was injected into the retro-orbital plexus of briefly anesthetized mice (isoflurane 2%). Blood samples from non-restrained, awake mice were collected by tail snip at different time intervals for a total duration of 75 minutes (8 samples/mouse). FITC-Inulin fluorescence was determined in both standards and samples by a plate reader with excitation and emission of 490 and 520 nm. A standard curve was generated from the infused FITC-Inulin solution and GFR was calculated by employing a two-phase exponential decay function (SigmaPlot 12.0 software).

3.2.5 RNA Extraction from Kidney Samples

To ensure the optimal RNA yield as well as the reduction of lysate viscosity, a total of 30 mg tissue pulverized with liquid nitrogen was subjected to disruption and homogenization (flash sonication for 5 seconds). Total RNA was obtained by using the RNeasy Minikit (Qiagen, Valencia, MD, and USA) following manufacturer's instructions. To minimize genomic contamination, an on-column DNase digestion step was included in the protocol. Two micrograms of total RNA were reverse transcribed with a High Capacity

cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Reverse transcription was performed in a thermal cycler (C1000^{TM}, Bio Rad) under the following parameters: 25 C for 10 minutes, 37 C for 120 minutes, followed by 85 C for 5 minutes.

3.2.6 Quantitative Real -Time Polymerase Chain Reaction Analysis

To quantify the expression levels of genes of interest, TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used. Reaction was performed in single micro capillary tubes (20 microliters volume) on a LightCycler (Roche Diagnostic, Laval, QC, Canada) for control and target gene expression primer probes (TaqMan® Gene Expression Assay, Applied Biosystems, Tab. 3.1). Six serial dilutions (1:10) of each target and reference genes served as a standard curve and was assayed together with the corresponding unknown samples on each plate. The quantitative real-time PCR profile was 95 C/15 seconds denature, 58 C/1 minute anneal-extension for 40 cycles. Reactions were performed in triplicate and delta-delta Ct (ΔΔCt) method was employed to determine the fold difference ($2^{-\Delta\Delta Ct}$) (Applied Biosystems).

Table 3.1. Primer probe information for quantitative real-time PCR

Target Primer	Probe ID
Grk2/Adrbk1	Mm00804778_m1
Grk3/Adrbk2	Mm00622042_m1
GRK4	Mm01213690_m1
GRK5	Mm00517039_m1
GRK6	Mm00442425_m1
Collagen	Mm00801606_m1
Target Primer	**Probe ID**

Sox6	Mm00488393_m1
Renin	Mm02342887_mH
Angiotensinogen	Mm00599662_m1
AT_1R	Mm00475056_m1
β_2AR	Mm01242584_m1
α_1dAR	Mm01328600_m1
$5\text{-}HT_1B$	Mm00439377_s1
$5\text{-}HT_2B$	Mm00434123_m1
D_1R	Mm02620146_s1
ACTB	Mm02619580_g1

3.2.7 Losartan Treatment-Experimental Design

The shGRK2 (+/-) transgenic mice were divided into three age-based groups (3, 6, and 12 month-old) with a number of six mice/group. Age-matched wild type C57Bl/6 mice (Charles River Laboratories) were used as a control. Treatment groups received 100 mg/kg/day Losartan (Sigma Aldrich) in drinking water for one month. Water alone was used as a control for all groups of mice. Water intake was measured daily and was approximately 25 ml/week/mouse. Mice were housed on a 12:12 hour light-dark cycle at 24 C, with free access to food and water. Systolic and diastolic blood pressures were measured weekly during the one-month treatment period using the non-invasive CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT). As a baseline, blood pressure before and three weeks after the treatment (washout period) was recorded.

To eliminate the influence of circadian rhythm, blood pressure was measured at the same time of day.

3.2.8 Plasma Renin Analysis

Plasma renin was measured by an enzyme-linked immunosorbent ELISA assay (Mouse Ren1-ELISA Kit, Sigma Aldrich) following manufacturer's instructions. The optical density at 450 nm was determined for each well using an Infinite M200 (TECAN) plate reader. Each measurement was done in triplicate and the mean absorbance for standards, controls and samples was calculated. Standard curve was generated with SigmaPlot software.

3.2.9 Connective Tissue Staining

In brief, mice were anesthetized with ketamine/xylazine (100/10 mg/kg intraperitoneal), exsanguinated by saline perfusion and directly perfused with 4% paraformaldehyde solution via vascular system. Paraffin-fixed tissues were sectioned (4 μm tick) and placed on positively-charged microscope slides. After deparaffinization and rehydration through serial decreases of ethanol concentration (100%, 95%, and 70%), slides were subjected to Masson's trichrome staining (Trichrome Stain-Kit, Abcam, Cambridge, MA, USA) following manufacturer's instructions. Interstitial collagen deposition was visualized by using a bright-light microscope (20X magnification) and image capture software (Leica DM RBE, Image Pro Plus 4.5.1 software).

3.2.10 H&E Staining

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin (200 μl/slide, Sigma Aldrich) for 5 minutes and eosin (400 μl/slide, Sigma Aldrich) for 30 seconds. Then slides were quickly dehydrated (2 changes of 100% ethanol), cleared in xylene, and mounted. Assessment of glomerular number was performed by using the point selection

tool with auto measure of Image J software (Bethesda, MD, USA). The mean glomerular number per unit area of tissue section (mm^2) was calculated from a total number of 160 microscopic fields for both samples and controls.

3.2.11 Immunohistochemistry of Renin

Renin was detected in paraffin-embedded kidney sections (3 μm tick) using a goat anti-mouse polyclonal antibody (15μg/ml, R&D Systems, Minneapolis, MN, USA). Tissue was stained with an anti-goat HRP-DAB tissue staining kit (CTS0008, R&D Systems) and counterstained with haematoxylin. In order to identify non-specific staining of the secondary antibodies, incubation buffer with no primary antibodies was used as a negative control. To reduce non-specific hydrophobic interactions between the primary antibodies and tissue, slides were incubated with a goat serum blocking reagent. To block binding to endogenous biotin, samples were incubated with avidin blocking reagent and then to block subsequent binding to the avidin, a biotin blocking reagent was used. A bright-field illumination microscope (20X magnification) was used to visualize tissue staining.

3.2.12 Statistical Analysis

Statistical differences between groups were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA, USA). All parametric data were analyzed with either Student's t test or one-way ANOVA followed by Bonferroni post hoc comparisons. SigmaPlot software version 12.1 (Cincinnati, OH, USA) was used for regression analysis. Statistical significance between mean values were determined based on $p < 0.05$ criterion.

3.3 Results

3.3.1 Effects of GRK2 Knockdown on Body Fat

To assess the impact of altered GRK2 expression on blood pressure regulation, we used a transgenic mouse model of systemic downregulation of GRK2 protein expression. The GRK2 (+/-) transgenic mice are characterized by a lean phenotype since CT analysis of the whole body scan revealed a significant increase in the lean tissue associated with a significant reduction of the adipose mass when compared with control wild type C57Bl/6 mice (Fig. 3.1A, 3.1B, and 3.1C). In addition, analysis of retroperitoneal fat, a well-defined tissue showed more than 60% reduction in shGRK2 mice (Fig. 3.1D).

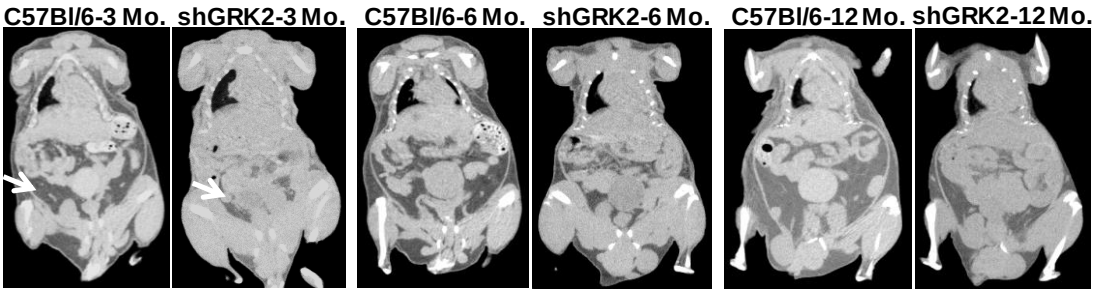
3.3.2 Effects of GRK2 Knockdown on Kidney Morphometry and Function

The organ weight-to-body weight ratios (heart, lung, and liver) showed no significant differences between the shGRK2 and control group with the exception of kidneys. A significant decrease in kidney weight of shGRK2 mice was recorded at 6-weeks, 12-weeks, and 6-months of age. There was no statistical significance with respect to the mass and length of both (left-right) shGRK2 kidneys. However, there was a significant difference between the mean renal length of shGRK2 mice when compared with age-matched C57Bl/6 mice (Fig. 3.2A, 3.2B, and 3.2C). Measurements of glomerular filtration rate after a single bolus administration of fluorescent-labelled inulin exhibited characteristics of a two-compartment system with an initial rapid decay phase, followed by a slower decay phase. By using nonlinear regression, the plasma elimination of FITC-inulin yielded a GFR of 232.21 ± 46.75 µl/min for control wild type mice, and a GFR of 76.39 ± 20.79 µl/min for shGRK2 mice (Fig. 3.2D).

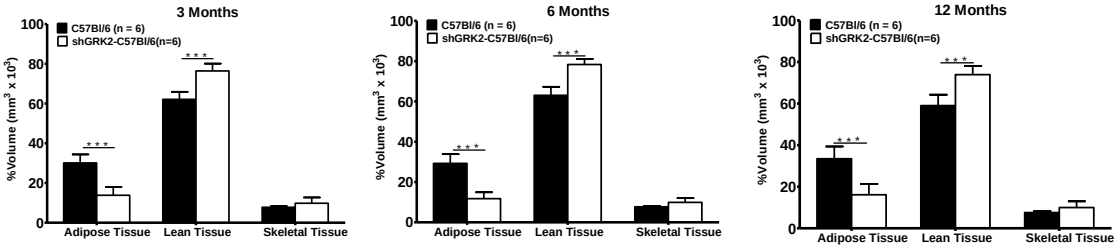
Figure 3.1: Characterization of shGRK2 mice phenotype

(A) Representative three-dimensional CT images of the whole body scan of shGRK2 and control C57Bl/6 mice outlining the difference in adipose and lean tissue. **(B)** and **(C)** Comparative quantitative analysis of adipose, lean, and skeletal tissue in shGRK2 and control mice. **(D)** Comparative quantitative analysis of retroperitoneal fat in shGRK2 and control mice at different weeks of age. Data represents the mean ± SD (n = 6). (***) Significant at ($p < 0.001$).

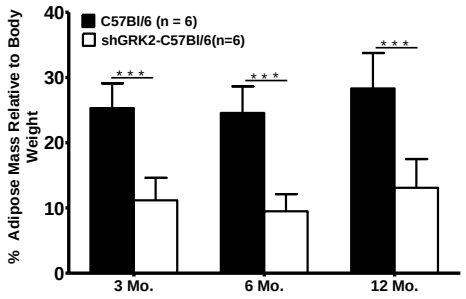
A)


C57Bl/6-3 Mo.
shGRK2-3 Mo.
C57Bl/6-6 Mo.
shGRK2-6 Mo.
C57Bl/6-12 Mo.
shGRK2-12 Mo.

B)


3 Months
6 Months
12 Months
C57Bl/6 (n = 6)
shGRK2-C57Bl/6(n=6)
%Volume (mm³ x 10³)
Adipose Tissue
Lean Tissue
Skeletal Tissue
***

C)


C57Bl/6 (n = 6)
shGRK2-C57Bl/6(n=6)
% Adipose Mass Relative to Body Weight
3 Mo.
6 Mo.
12 Mo.
***

D)

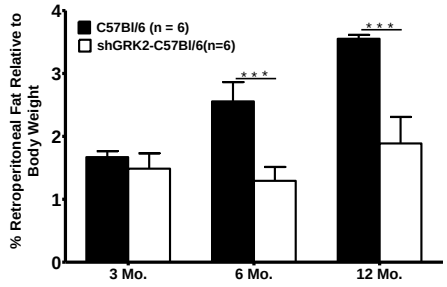
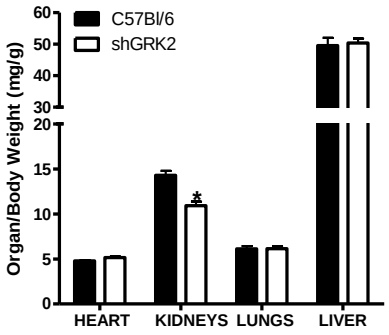

C57Bl/6 (n = 6)
shGRK2-C57Bl/6(n=6)
% Retroperitoneal Fat Relative to Body Weight
3 Mo.
6 Mo.
12 Mo.
***

Figure 3.2: Effects of GRK2 knockdown on kidney anatomy and function

(A) Quantification of organ mass relative to body weight in 3 month-old shGRK2 and C57Bl/6 mice. **(B)** Digital images of wild-type versus shGRK2 kidneys outing the difference in size. **(C)** Quantification of kidney mass (right & left) relative to tibia length at different months of age. Data represents the mean ± SD of 6 mice. (***) Indicates statistically significant differences compared to control, age-matched C57Bl/6 mice ($p < 0.001$). **(D)** Renal inulin clearance (µl/minute) of shGRK2 and control C57Bl/6 mice under basal conditions. Data represents the mean ± SD. (*) Significant at ($p < 0.05$); (**) Significant at ($p < 0.01$); (***) Significant at ($p < 0.001$).

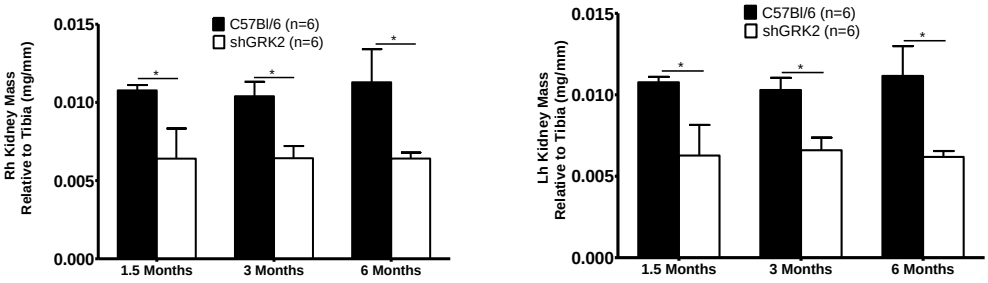
A)


C57Bl/6
shGRK2
Organ/Body Weight (mg/g)
60
50
40
30
20
15
10
5
0
*
HEART
KIDNEYS
LUNGS
LIVER

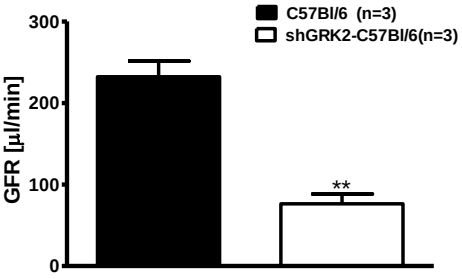
B)


Wild-type
1.5 months
shGRK2
1.5 months
Wild-type
3 months
shGRK2
3 months
Wild-type
6 months
shGRK2
6 months

C)


C57Bl/6 (n=6)
shGRK2 (n=6)
Rh Kidney Mass
Relative to Tibia (mg/mm)
0.015
0.010
0.005
0.000
*
1.5 Months
3 Months
6 Months
C57Bl/6 (n=6)
shGRK2 (n=6)
Lh Kidney Mass
Relative to Tibia (mg/mm)
0.015
0.010
0.005
0.000
*
1.5 Months
3 Months
6 Months

D)


C57Bl/6 (n=3)
shGRK2-C57Bl/6(n=3)
GFR [µl/min]
300
200
100
0
**

To assess the effect of GRK2 knockdown on kidney function, semi-quantitative analysis of urinary content was performed and a moderate increase in protein expression level in urine collected from 1, 3, and 6 month-old shGRK2 mice was observed when compared with age-matched control mice (Tab. 3.2).

Quantitative analysis of urine revealed a significant increase in total protein content in urine of 3 month-old shGRK2 mice when compared with control (13.5 ± 1.7 vs. 7.2 ± 2 g/l, $p < 0.001$). As well as an increase in blood urea nitrogen was recorded (794 ± 29 vs. 732 ± 28 mg/dl, $p < 0.001$). The urinary electrolyte excretion was measured and a significant decreased in urinary sodium content was observed for shGRK2 mice across the all age-groups analyzed (Tab. 3.3).

With respect to blood biochemistry (Tab. 3.4), no plasma sodium variation was observed among the all shGRK2 groups analyzed while an increase in potassium (5.2 ± 0.5 vs. 3.9 ± 0.38 mmol/l, $p < 0.001$) and phosphorous (8.5 ± 1.2 vs. 7 ± 0.8 mg/dl, $p < 0.05$) can be observed in 6 month-old shGRK2 mice. In addition, an increased in blood urea was recorded in plasma of shGRK2 mice when compared with control wild type mice (28 ± 1.4 vs. 22.7 ± 2 mg/dl, $p < 0.001$). Our data also indicate low serum albumin levels for shGRK2 mice (25 ± 2.3 vs. 27 ± 2.9 g/l, $p < 0.001$).

Table 3.2: Semi-quantitative analysis of urine

Samples were collected from shGRK2 and control C57Bl/6 mice (n = 6) and assessed by urine analysis test strip. Expression level: negative (absent), modest (+), moderate (++).

Analyte (unit)	1 month (n=6)		3 months (n=6)		6 months (n=6)	
	wt	shGRK2	wt	shGRK2	wt	shGRK2
pH	5	6	5	7	6	5
Nitrate	Negative	Negative	Negative	Negative	Negative	Negative
Protein	Negative	Positive(+)	Negative	Positive(++)	Positive(+)	Positive(++)
Glucose	Negative	Negative	Negative	Negative	Negative	Negative
Ketones	Negative	Negative	Negative	Negative	Negative	Positive(+)
Bilirubin	Negative	Negative	Negative	Negative	Negative	Negative
Leucocytes	Negative	Negative	Negative	Negative	Negative	Negative
Blood	Negative	Negative	Negative	Negative	Negative	Negative
Hemoglobin	N/A	N/A	N/A	N/A	N/A	N/A

Table 3.3: Biochemical analysis of urine

Samples were collected from shGRK2 and control C57Bl/6 mice. Data represents the mean ± SD (n=6). (*) Significant at ($p < 0.05$); (**) Significant at ($p < 0.01$); (***) Significant at ($p < 0.001$).

Analyte (unit)	1 month (n=6)		3 months (n=6)		6 months (n=6)	
	wt	shGRK2	wt	shGRK2	wt	shGRK2
Sodium (mmol/L)	199 ± 46	149 ± 44	214 ± 29	150 ± 22(*)	175 ± 30	105 ± 53(**)
Potassium (mmol/L)	72 ± 23	87 ± 4	91 ± 6	104 ± 14	94 ± 6	83 ± 14
Chloride (mmol/L)	198 ± 60	232 ± 61	232 ± 43	245 ± 56	233 ± 31	165 ± 66
Phosphorous (mg/dL)	47 ± 3	46 ± 0.4	41 ± 3	47 ± 2	47 ± 1	46 ± 0.5
Calcium (mg/dL)	5.2 ± 1.2	4.9 ± 1.3	5.2 ± 1.3	6.6 ± 1.4	5.1 ± 0.2	5.9 ± 0.01
Total Protein (g/L)	2.2 ± 0.07	2.8 ± 0.6	7.2 ± 2.3	13.5 ± 1.7(***)	12.2 ± 2.4	12.9 ± 0.9
Albumin (g/L)	0.15 ± 0.1	0.1 ± 0.01	0.7 ± 0	0.5 ± 0.34	0.6 ± 0.1	0.7 ± 0.05
Blood Urea (mg/dL)	823 ± 24	837 ± 9	732 ± 28	794 ± 29(***)	791 ± 11	788 ± 16
Creatinine (mg/dL)	25 ± 12	26 ± 5	37 ± 4	30 ± 16	38 ± 2	40 ± 1

Table 3.4: Biochemical analysis of blood

Samples were collected from shGRK2 and control C57Bl/6 mice. Data represents the mean ± SD (n=6). (*) Significant at ($p < 0.05$); (**) Significant at ($p < 0.01$); (***) Significant at ($p < 0.001$).

Analyte (unit)	1 month (n=6)		3 months (n=6)		6 months (n=6)	
	wt	shGRK2	wt	shGRK2	wt	shGRK2
Sodium (mmol/L)	149 ± 0.4	147 ± 2.1	149 ± 3.2	151 ± 2.0	150 ± 2.8	146 ± 8.9
Potassium (mmol/L)	4.4 ± 0.10	4.1 ± 0.07	4.0 ± 0.32	4.1 ± 0.61	3.9 ± 0.38	5.2 ± 0.5(***)
Chloride (mmol/L)	114 ± 1.2	111 ± 1.6	113 ± 2.8	118 ± 2(*)	113 ± 2.4	113.2 ± 8.4
Phosphorous (mg/dL)	10.5 ± 0.4	9.6 ± 0.4	7.5 ± 1.2	7.8 ± 1.7	7.0 ± 0.8	8.5 ± 1.2(*)
Calcium (mg/dL)	9.6 ± 0.3	9.9 ± 0.1	8.9 ± 0.3	8.9 ± 0.2	9.1 ± 0.2	9.2 ± 0.4
Total Protein (g/L)	44.2 ± 1.3	44 ± 0.3	47.0 ± 3.2	45 ± 2.3	49.2 ± 1.7	49.1 ± 1.7
Albumin (g/L)	26 ± 1.0	26.3 ± 0.7	27.0 ± 1.1	25.2 ± 0.7(*)	27.0 ± 1.5	25.1 ± 2.3(*)
Blood Urea (mg/dL)	22.3 ± 2.0	19.1 ± 1.3(**)	24.7 ± 3.4	28.6 ± 2.67(**)	22.7 ± 2.9	28.0 ± 1.4(***)
Creatinine (mg/dL)	0.22 ± 0.02	0.18 ± 0.01(**)	0.22 ± 0.01	0.18 ± 0.03(**)	0.19 ± 0.01	0.20 ± 0.01
Glucose (mg/dL)	213 ± 43	252 ± 26	219 ± 38	234 ± 50	270 ± 38	256 ± 45
Total Cholesterol (mg/dL)	95 ± 6.6	92 ± 8.5	91 ± 18	90 ± 10.5	106 ± 17	89 ± 8.0(*)
Triglycerides (mg/dL)	148 ± 30	131 ± 13	141 ± 52	194 ± 97	171 ± 62	172 ± 41
HDL-Cholesterol (mg/dL)	44.7 ± 2.9	47.1 ± 3.0	53.0 ± 9.1	51.7 ± 3.7	59.4 ± 8.8	48.4 ± 3.6(***)
LDL-Cholesterol (mg/dL)	7.5 ± 0.2	10.1 ± 0.6(***)	2.8 ± 0.6	4.5 ± 0.1(***)	3.2 ± 0.6	4.0 ± 0.6(**)

3.3.3 Effects of GRK2 Knockdown on GPCRs Expression in Kidney

Microarray analysis confirmed that GRK2 gene expression is down-regulated in kidney tissue of the transgenic mice. Consistent with previous observations that GRK2 down-regulation increased lipolysis, our data indicate that several genes with role in lipid metabolism are up-regulated: carboxylesterase gene with role in cholesterol hydrolysis, adiponectin with role in lipid catabolism, and Apolipoprotein E which is a lipid transporter and is the major ligand for LDL receptors (Tab. 3.5). One of the question to address was whether GRK2 inhibition leads to up-regulation of other members of GRKs family as a compensatory mechanism. In this regard, we found out that while GRK2 mRNA expression is constantly maintained at decreased levels over time, GRK3, GRK4, and GRK5 are significantly increased in kidney tissue collected from 3 and 6 month-old shGRK2 mice (Fig. 3.3A). To assess whether alteration in GRK2 level have an impact on the expression status of GPCRs, the mRNA level of several GPCRs with critical role in blood pressure regulation was measured.

We found a significant shift/decrease in the mRNA expression of dopamine type 1 receptor (D_1R) in kidney collected from shGRK2 mice when compared with age-matched control. Dopamine interacts with AT_1Rs and α_1ARs in the proximal tubule to maintained sodium homeostasis and any defect in dopaminergic system associates with an opposed action of AT_1R. Indeed, our data indicate a significant increase in both AT_1R and α_1ARs mRNA expression in shGRK2 kidney. In addition, a distinct expression pattern of serotonin 5-HT receptors was observed: while both types of receptors were significantly increased in shGRK2 kidney tissues, the HT-1bR reach a peak of expression at 1 month

Table 3.5: Microarray analysis of gene expression in kidney tissue

Top 25 significantly **(A)** increased and **(B)** decreased genes in kidneys harvested from shGRK2 vs. C57Bl/6 mice at 3 months of age.

A)

GENE	PROTEIN NAME	Fold Change
Slc7a12	Solute carrier family 7	8.304
Bcl6	B cell leukemia/lymphoma 6	5.201
Prlr	Prolactin receptor	4.781
Prlr	Prolactin receptor	3.697
Ces1	Carboxyl esterase	2.962
Kynu	Kynureninase	2.736
Upk1b	Uroplakin 1B	2.581
Cfd	Complement factor D (adipsin)	2.548
BC089597	unknown	2.336
Adipoq	adpipnectin	2.167
Abcb1b	ATP binding cassette sub-family 1B	2.163
Cfi	Compliment factor 1	2.070
Hao3	hydroxyacid oxidase 2	2.020
Gsdmc2	gasdermin 2	1.973
Apoe	Apolipoprotein E	1.963
Cyp2c44	cytochrome P450 2C	1.942
Bhmt	betaine-homocysteine S-methyltransferase	1.918
Bbox1	butyrlbetaine hydroxylase 1	1.915
Thrsp	thyroid hormone responsive	1.907
Cidec	cell death-inducing DFFA-like effector c	1.781
Bhlhe41	basic helix-loop-helix family member e41	1.764
Krt18	keratin 18	1.747
Gm129	circadian associated repressor of transcription	1.710
Gsta4	Glutathione S-transferase alpha 1	1.694
Abcc9	ATP-binding cassette C9	1.688

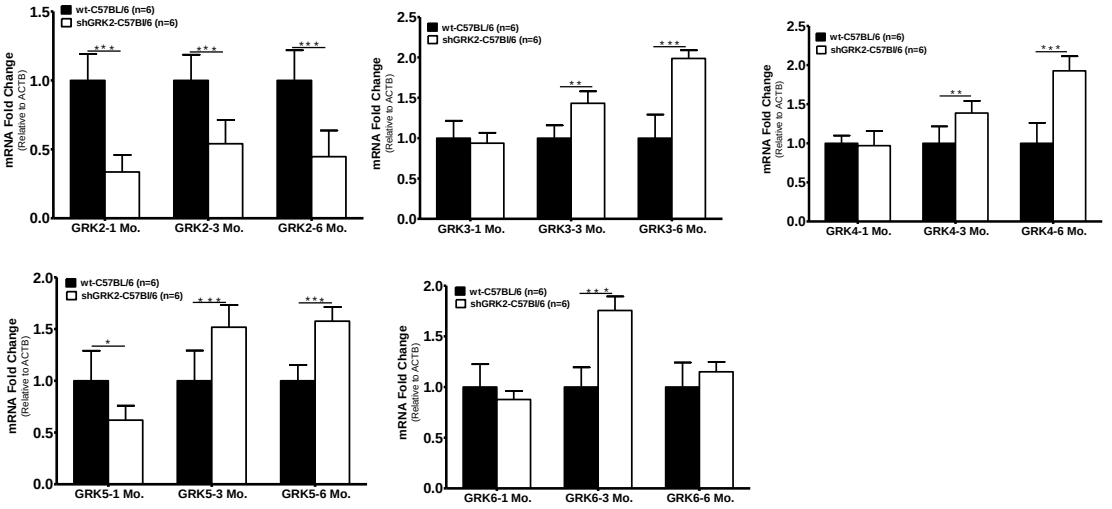
B)

GENE	PROTEIN NAME	Fold Change
Ctxn3	cortexin 3	-4.187
EG633640	predicted gene	-3.550
Pon2	paraoxanase 2	-3.345
Synpr	synaptoporin	-2.786
Adrbk1	GRK2	-2.486
Akr1c18	aldo-keto reductase family C18	-2.418
Igj	immunoglobin joining chain	-2.302
GusB	Glucuronidase beta	-2.299
Cyp2b10	cytochrome P4502b10	-2.258
Popdc3	popeye domain containing 3	-2.243
Slc22a7	solute carrier family 22a7	-2.066
Zdhhc2	Zinc finger DHHC domain containing 2	-2.000
Mpped1	metalophosphoesterase domain containing 1	-1.903
Hsd11b1	11 beta dehydrogenase 1	-1.885
Tpk1	thiamine pryophosphokinase	-1.856
Pi4k2b	phosphatidylinositol 4-kinase type 2 beta	-1.822
Rnf180	ring finger protein 180	-1.782
Sectm1b	secreted and transmembrane 1B	-1.765
Igl-v1	immunoglobulin lambda variable 1	-1.763
Mfsd2	major facilitator superfamily domain containing 2a	-1.761
Nt5dc2	5'-nucleotidase domain containing 2	-1.752
Trib2	tribbles homology 2	-1.752
Pank1	pantothenate kinase 1	-1.743
Hspb8	heat shock protein 8	-1.732
Csgalnact1	chondroitin sulfate N-acetylgalactosaminyl transferase 1	-1.734

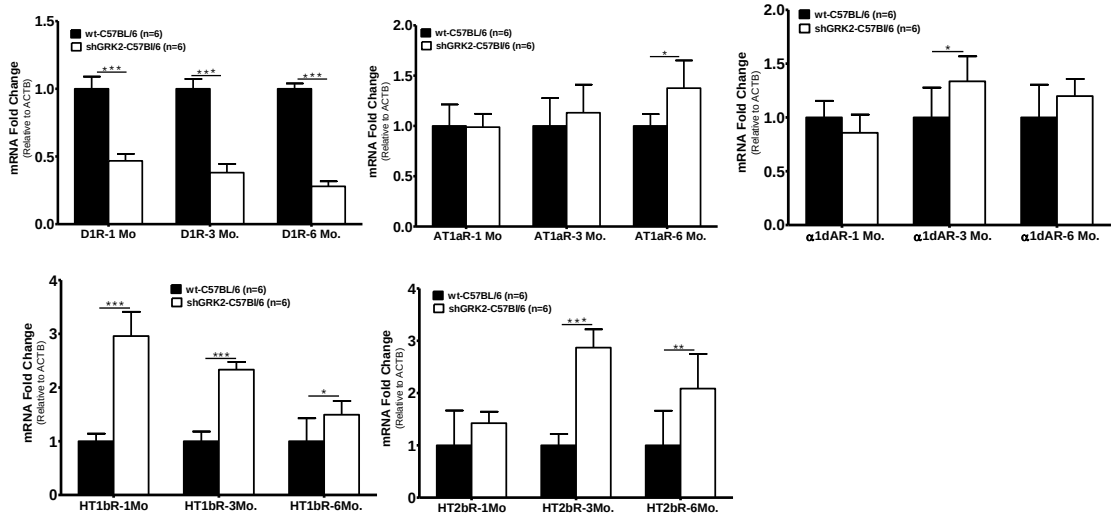
Figure 3.3: Effects of GRK2 knockdown on GRKs expression in kidney

(A) Quantitative RT-PCR analysis of GRK 2, 3, 4, 5 and 6 mRNA expressions in kidney. Expressions levels are normalized to actin (ACTB). **(B)** Quantitative RT-PCR analysis of several GPCRs mRNA expression in kidney. Expressions levels are normalized to ACTB. Data represents the mean ± SD (n=6). (*) Significant at ($p < 0.05$); (**) Significant at ($p < 0.01$); (***) Significant at ($p < 0.001$).

A)


wt-C57BL/6 (n=6)
shGRK2-C57Bl/6 (n=6)
mRNA Fold Change
(Relative to ACTB)
GRK2-1 Mo.
GRK2-3 Mo.
GRK2-6 Mo.
GRK3-1 Mo.
GRK3-3 Mo.
GRK3-6 Mo.
GRK4-1 Mo.
GRK4-3 Mo.
GRK4-6 Mo.
GRK5-1 Mo.
GRK5-3 Mo.
GRK5-6 Mo.
GRK6-1 Mo.
GRK6-3 Mo.
GRK6-6 Mo.

B)


wt-C57BL/6 (n=6)
shGRK2-C57Bl/6 (n=6)
mRNA Fold Change
(Relative to ACTB)
D1R-1 Mo
D1R-3 Mo.
D1R-6 Mo.
AT1aR-1 Mo
AT1aR-3 Mo.
AT1aR-6 Mo.
α1dAR-1 Mo.
α1dAR-3 Mo.
α1dAR-6 Mo.
HT1bR-1Mo
HT1bR-3Mo.
HT1bR-6Mo.
HT2bR-1Mo
HT2bR-3Mo.
HT2bR-6Mo.

of age and then slowly decreased as mice aged. On the hand, the expression of HT-2bR increased gradually with the development of high blood pressure (Fig. 3.3B).

3.3.4 Effects of GRK2 Knockdown on RAS Expression in Kidney

Our data indicate that the mRNA level of renal angiotensinogen increased significantly in shGRK2 mice during the development of high blood pressure with a peak at six months of age (Fig. 3.4A). In addition, the level of intra-renal renin mRNA expression increased gradually starting with the pre-hypertensive shGRK2 mice and this increase was still maintained at six months of age (Fig. 3.4B). On the other hand, no significant changes in plasma renin concentration were noticed in pre-hypertensive and 3 month-old shGRK2 mice when compared with age-matched control wild type mice. However, the level of renin emerged a significant increase in plasma of shGRK2 mice at six months of age (Fig. 3.4C). In addition, renin mRNA expression correlates positively with renal renin protein expression as visualized by immunochemistry staining (Fig. 3.4D).

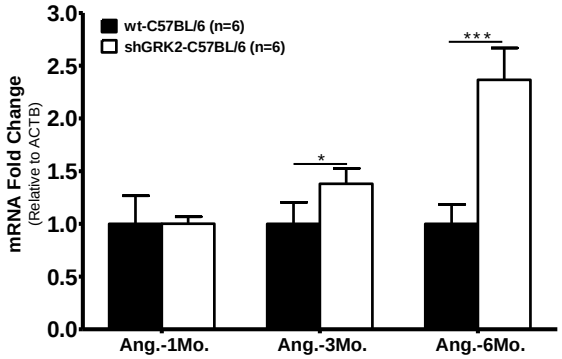
3.3.5 Effects of AT_1R Blocker on Blood Pressure and Kidney Function

To verify the involvement of AT_1R axis in blood pressure modulation of the shGRK2 mice, a selective AT_1R blocker losartan was given orally (100mg/kg/day) to our shGRK2 and wild type C57Bl/6 mice for four weeks. Water alone was used as a control. A gradual drop in both systolic and diastolic blood pressures was recorded over the entire duration of treatment for all aged shGRK2 groups analyzed (Fig 3.5A).

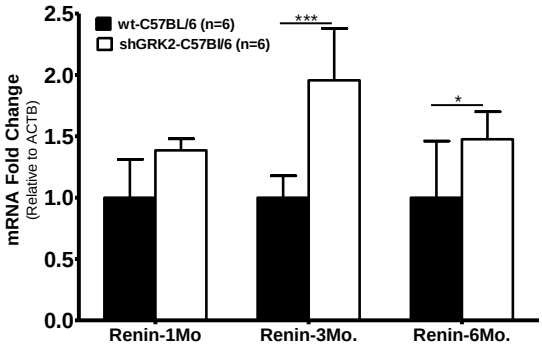
Figure 3.4: Effects of GRK2 knockdown on RAS

Quantitative RT-PCR analysis of angiotensinogen **(A)** and renin **(B)** mRNA expressions in kidney of shGRK2 and control C57Bl/6 mice (n=6). Expressions levels are normalized to ACTB. **(C)** Plasma renin expression in shGRK2 and control mice as measured by enzyme-linked immunosorbent ELISA assay. Standard curve as obtained by regression analysis (r2 = 0.99). Data represents the mean ± SD (n=6). (*) Indicates statistically significant differences compared to control ($p < 0.05$). **(D)** Representative immunostaining of renin protein expression in kidney sections of shGRK2 mice vs. control wild type.

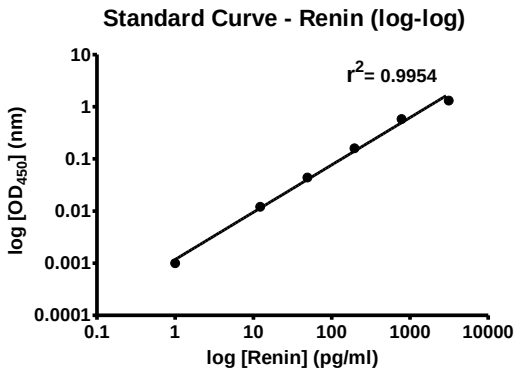
A)

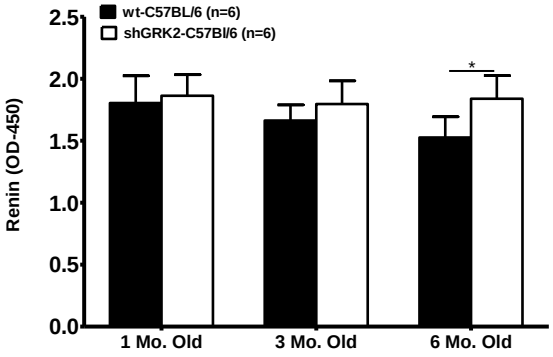

wt-C57BL/6 (n=6)
shGRK2-C57BL/6 (n=6)
mRNA Fold Change
(Relative to ACTB)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
***
Ang.-1Mo.
Ang.-3Mo.
Ang.-6Mo.

B)


wt-C57BL/6 (n=6)
shGRK2-C57Bl/6 (n=6)
mRNA Fold Change
(Relative to ACTB)
2.5
2.0
1.5
1.0
0.5
0.0
***
*
Renin-1Mo
Renin-3Mo.
Renin-6Mo.

C)


Standard Curve - Renin (log-log)
r2= 0.9954
log [OD450] (nm)
10
1
0.1
0.01
0.001
0.0001
0.1
1
10
100
1000
10000
log [Renin] (pg/ml)


wt-C57BL/6 (n=6)
shGRK2-C57Bl/6 (n=6)
Renin (OD-450)
2.5
2.0
1.5
1.0
0.5
0.0
*
1 Mo. Old
3 Mo. Old
6 Mo. Old

D)

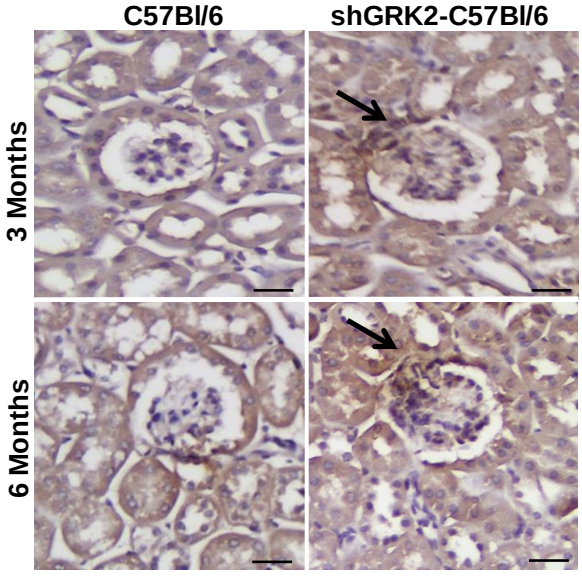
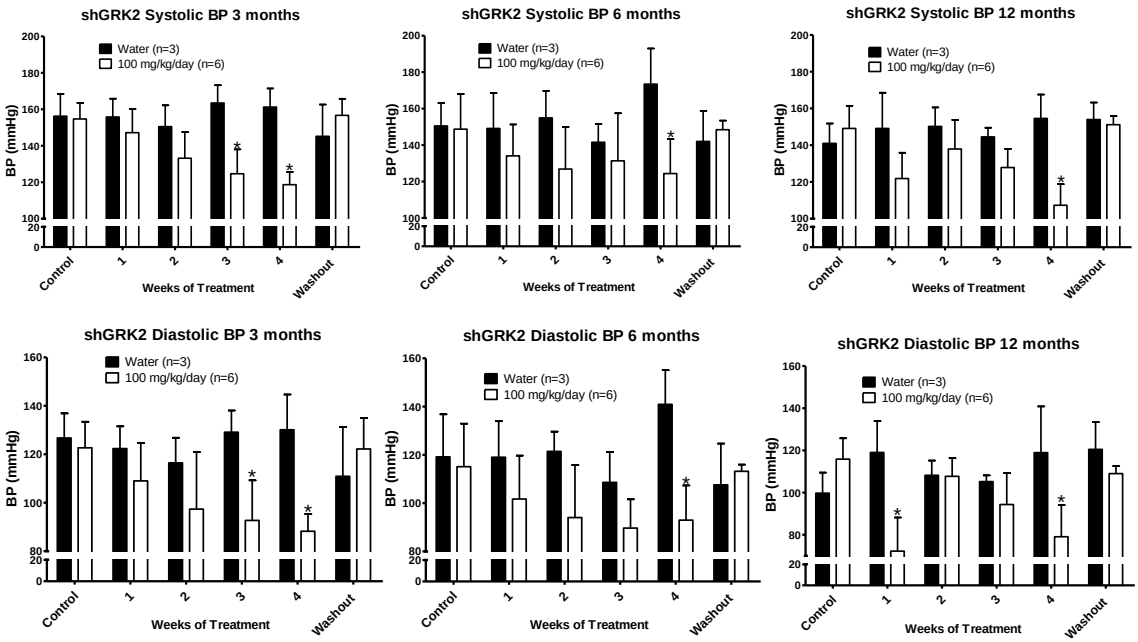

C57Bl/6
shGRK2-C57Bl/6
3 Months
6 Months

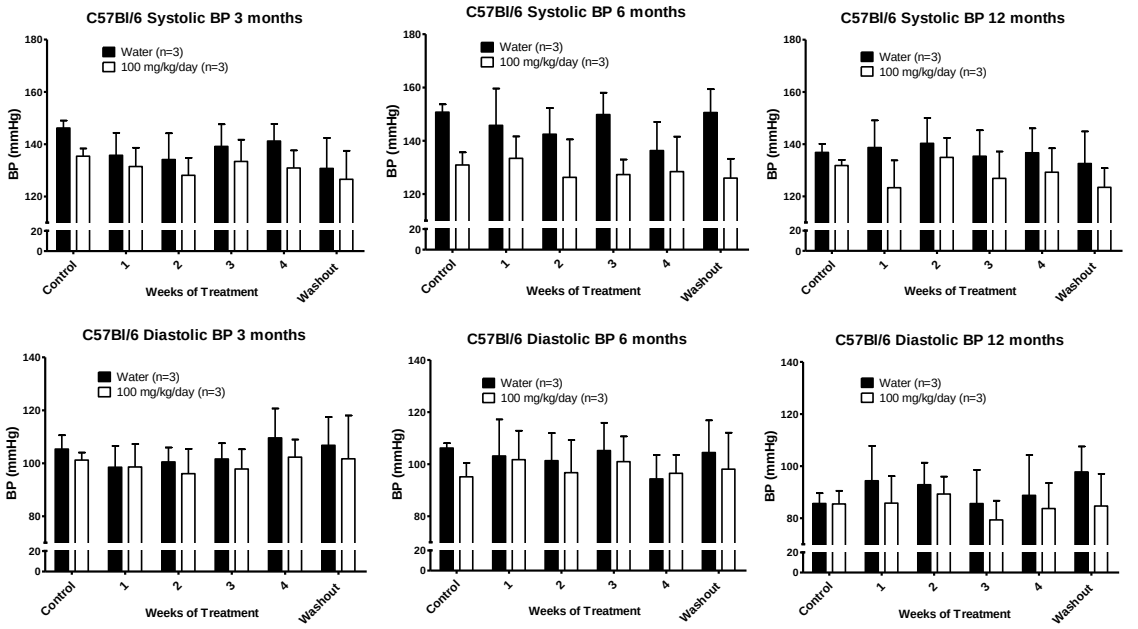
Figure 3.5: Effects of AT_1R specific blocker Losartan on blood pressure

(A) Systolic pressures, diastolic pressures, and heart rates in response to 100 mg losartan/kg/day and water control in shGRK2. Data represents the mean ± SD (n=9). (*) Indicates statistically significant differences compared to control ($p< 0.05$). **(B)** Systolic pressures, diastolic pressures, and heart rates in response to 100 mg losartan/kg/day and water control in C57Bl/6. Data represents the mean ± SD (n=6).

A)


shGRK2 Systolic BP 3 months
shGRK2 Systolic BP 6 months
shGRK2 Systolic BP 12 months
shGRK2 Diastolic BP 3 months
shGRK2 Diastolic BP 6 months
shGRK2 Diastolic BP 12 months
Water (n=3)
100 mg/kg/day (n=6)
BP (mmHg)
Control
1
2
3
4
Washout
Weeks of Treatment

B)


C57Bl/6 Systolic BP 3 months
C57Bl/6 Systolic BP 6 months
C57Bl/6 Systolic BP 12 months
C57Bl/6 Diastolic BP 3 months
C57Bl/6 Diastolic BP 6 months
C57Bl/6 Diastolic BP 12 months
Water (n=3)
100 mg/kg/day (n=3)
BP (mmHg)
Control
1
2
3
4
Washout
Weeks of Treatment

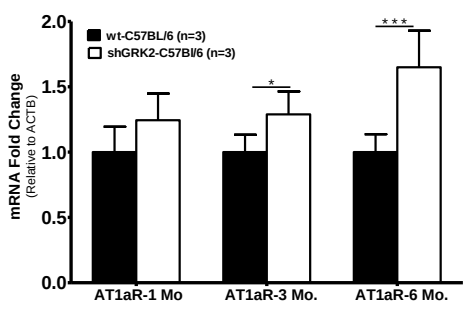
Blood pressure of the shGRK2 mice decreased significantly by the end of four weeks of treatment when compared with water control (116/86 ± 12/15 vs. 163/130 ± 14/16 mmHg, $p < 0.01$). No significant changes in blood pressures were observed for wild type mice regardless if mice were on losartan treatment or water (119/84 ± 9/7 vs. 128/84 ± 8/12 mmHg) (Fig. 3.5B).

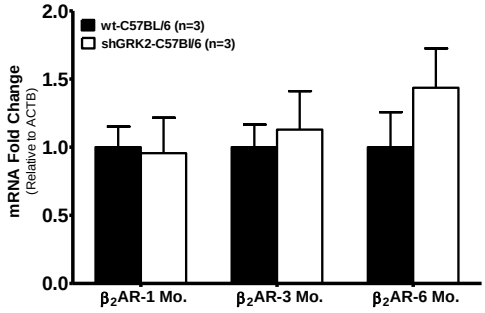
After a three-week wash out period, blood pressure recovered its pre-treatment value supporting the observation that AT_1R-mediated signaling is responsible for the blood pressure elevation of the shGRK2 mice (152/114 ± 6/5 vs. 157/112 ± 8/6 mmHg). Since the mRNA level of AT_1 receptors is increased in kidney of shGRK2 mice (Fig. 3.6A), we examined the effect of losartan on its expression and a significant decreased was recorded for shGRK2 mice when compared with control-water group. On the other hand, a more intricate pattern of expression was noticed for D_1R and β_2AR: while D_1R expression is unchanged and maintained at decreased levels, a pronounce increase in β_2AR mRNA expression was observed (Fig. 3.6B). In addition, a significant decreased in GRK2 and GRK5 mRNA expression levels were observed for shGRK2 mice in response to losartan treatment (Fig. 3.6C) while mRNA level of GRK3 and GRK4 expression was unchanged. Blockade of AT_1 receptor was associated with a significant increase in renin mRNA level (Fig. 3.6D). Our data also indicate that the inulin clearance rate of shGRK2 mice was rescued under losartan treatment to 178.29 ± 35.02 µl/min from 76.39 ± 20.79 µl/min, value measured under basal conditions (Fig. 3.6E).

Figure 3.6: Effects of AT_1R specific blocker Losartan on kidney

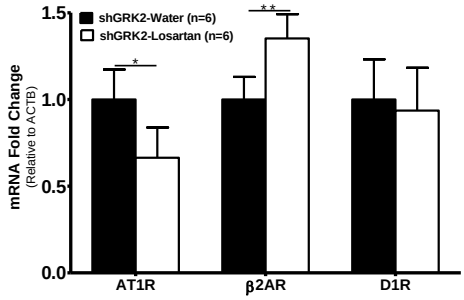
(A) Quantitative RT-PCR analysis of AT_1R and β_2AR mRNA expressions in aortic tissue of shGRK2 and control C57BL/6 mice. Expressions levels are normalized to ACTB **(B)** Quantitative RT-PCR analysis of AT_1R, β_2AR, and D_1R mRNA expressions in kidney collected from shGRK2 mice under basal and 4 weeks of losartan treatment (100mg/kg/day). Expressions levels are normalized to ACTB. **(C)** Quantitative RT-PCR analysis of GRK2, GRK3, GRK4, and GRK5 mRNA expressions in kidney collected from shGRK2 mice under basal and 4 weeks of losartan treatment (100mg/kg/day). Expressions levels are normalized to ACTB. **(D)** Quantitative RT-PCR analysis of angiotensinogen, renin, and $TGF\beta_1$ mRNA expressions in kidney of shGRK2 mice under basal and 4 weeks of losartan treatment (100mg/kg/day). Expressions levels are normalized to ACTB. **(E)** Renal inulin clearance (µl/minute) of shGRK2 mice under basal and 4 weeks of losartan treatment (100mg/kg/day). Data represents the mean ± SD (n=6). (*) Significant at ($p<0.05$); (**) Significant at ($p<0.01$); (***) Significant at ($p<0.001$).

A)

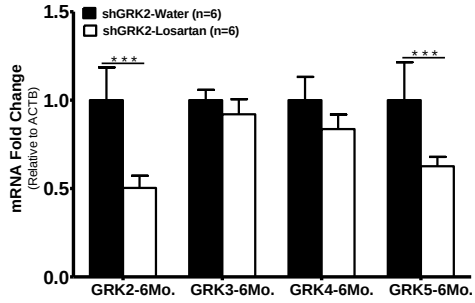

wt-C57BL/6 (n=3)
shGRK2-C57Bl/6 (n=3)
mRNA Fold Change
(Relative to ACTB)
2.0
1.5
1.0
0.5
0.0
*
***
AT1aR-1 Mo
AT1aR-3 Mo.
AT1aR-6 Mo.


wt-C57BL/6 (n=3)
shGRK2-C57Bl/6 (n=3)
mRNA Fold Change
(Relative to ACTB)
2.0
1.5
1.0
0.5
0.0
β2AR-1 Mo.
β2AR-3 Mo.
β2AR-6 Mo.

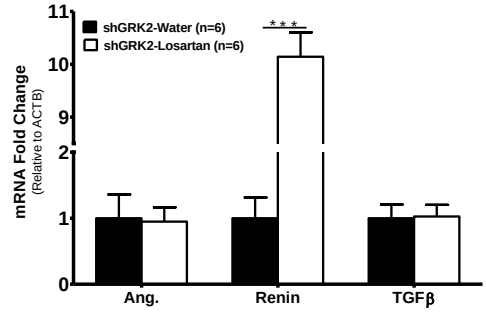
B)


shGRK2-Water (n=6)
shGRK2-Losartan (n=6)
mRNA Fold Change
(Relative to ACTB)
1.5
1.0
0.5
0.0
*
**
AT1R
β2AR
D1R

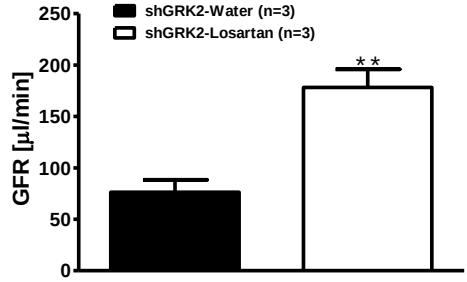
C)


shGRK2-Water (n=6)
shGRK2-Losartan (n=6)
mRNA Fold Change
(Relative to ACTB)
1.5
1.0
0.5
0.0
***
***
GRK2-6Mo.
GRK3-6Mo.
GRK4-6Mo.
GRK5-6Mo.

D)


shGRK2-Water (n=6)
shGRK2-Losartan (n=6)
mRNA Fold Change
(Relative to ACTB)
11
10
9
2
1
0
***
Ang.
Renin
TGFβ

E)


shGRK2-Water (n=3)
shGRK2-Losartan (n=3)
GFR [µl/min]
250
200
150
100
50
0
**

3.3.6 Effects of GRK2 Knockdown on Kidney Injury

One of the most common consequences of chronic hypertension is the injury of key target organs including kidney. Therefore, we examined the expression level of pro-fibrotic markers in kidney tissues of shGRK2 and age-matched wild type mice. We found that TGFβ_1, a key mediator of renal fibrosis through the activation of extracellular matrix proteins such as collagen, is increased at both protein and mRNA levels (Fig. 3.7A, 3.7B, and 3.7C). The expression level of TGFβ_1 increase significantly in 3-month old shGRK2 mice, age that correspond with onset of hypertension and this increase was still maintained at six months of age. Histological analysis of kidney sections revealed interstitial fibrosis sights in 6-month old shGRK2 mice. In addition, Masson' staining depicts increase collagen deposition around the arteries. The total glomerular number was significantly reduced in shGRK2 across the all age-analyzed when compared with control mice (Fig. 3.7D, 3.7E, and 3.7F).

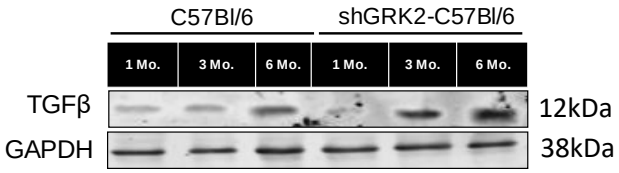
3.4 Discussion

The role of GRK2 in the embryonic development is essential since GRK2 gene ablation reveals that no mice embryos survive post-embryonic day E15.5 (Jaber *et al.*, 1996). GRK2 is expressed in multipotent and migratory cell populations during the early stages of embryogenesis. From embryonic day E15.5, GRK2 is expressed in several organs and tissues such as heart, liver, lung primordia and motor neurons supporting its broader role in embryogenesis (Sefton *et al.*, 2000). Recent evidences emphasize that GRK2 acts as a positive regulator of Hedgehog (Hh) signaling with crucial roles in embryonic development as well as physiological processes and many human disorders (Zhao *et al.*, 2016).

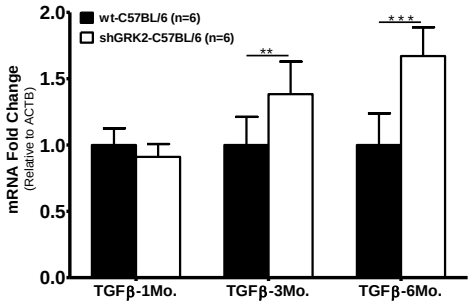
Figure 3.7: Effects of GRK2 knockdown on kidney

(A) Representative immunoblot for TGFβ$_1$ and GAPDH expression in kidney tissue obtained from wild-type C57Bl/6 and shGRK2 mice. Quantitative RT-PCR analysis of TGFβ$_1$**(B)** and collagen IV**(C)** mRNA expression in kidney of shGRK2 and control C57Bl/6 mice (n=6). Expressions levels are normalized to ACTB. **(D)** Representative histological images of kidney sections stained with H&E collected from shGRK2 and age-matched control mice. **(D)** Evaluation of glomerular number in H&E stained histological kidney sections. Glomerular number from 160 microscopic fields (20X) is reported as a mean per unit area of tissue sections. **(E)** Representative histological images of Masson' trichrome staining of kidney sections from shGRK2 and control mice. Blue represents collagen, red represents muscle fibers, and black nuclei.

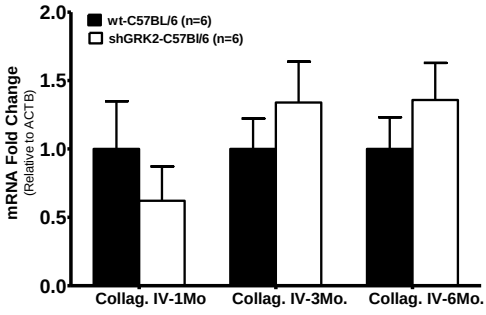
A)


C57Bl/6
shGRK2-C57Bl/6
1 Mo.
3 Mo.
6 Mo.
1 Mo.
3 Mo.
6 Mo.
TGFβ
GAPDH
12kDa
38kDa

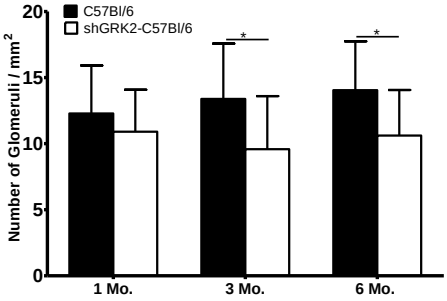
B)


wt-C57BL/6 (n=6)
shGRK2-C57BL/6 (n=6)
mRNA Fold Change
(Relative to ACTB)
2.0
1.5
1.0
0.5
0.0
**
***
TGFβ-1Mo.
TGFβ-3Mo.
TGFβ-6Mo.

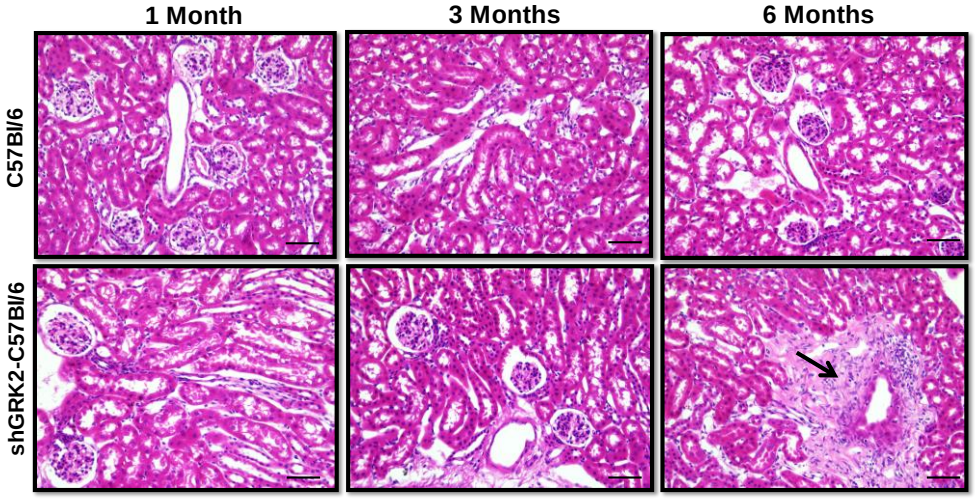
C)


wt-C57BL/6 (n=6)
shGRK2-C57Bl/6 (n=6)
mRNA Fold Change
(Relative to ACTB)
2.0
1.5
1.0
0.5
0.0
Collag. IV-1Mo
Collag. IV-3Mo.
Collag. IV-6Mo.

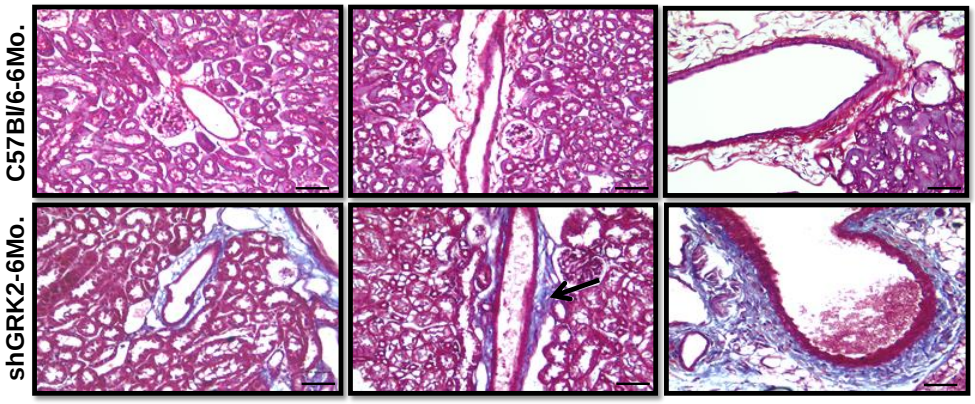
D)


C57Bl/6
shGRK2-C57Bl/6
Number of Glomeruli / mm2
20
15
10
5
0
*
*
1 Mo.
3 Mo.
6 Mo.

E)


1 Month
3 Months
6 Months
C57Bl/6
shGRK2-C57Bl/6

F)


C57Bl/6-6Mo.
shGRK2-6Mo.

In our transgenic mouse model, the targeted knockdown of GRK2 expression has translated into a growth-deficient phenotype with lower body mass indices and a significant decrease of adipose mass. Indeed, microarray analysis revealed that shGRK2 mice exhibit upregulations of several genes with role in lipid metabolism and this supports the past observations that GRK2 plays an important role in the regulation of adiposity (Vila-Bedmar *et al.*, 2012). Otherwise, our GRK2 heterozygote mice developed normally, with no difference in organ weight-to-body weight ratio, the only exception being the kidneys that were characterized by significant decreases in size and weight, as recorded at both birth and adulthood. However, the reason for which shGRK2 mice had a kidney phenotype with no other organ abnormalities remains to be determined.

The kidney has a central role among the organs with role in blood pressure homeostasis. Among the various cellular defects associated with the inception of hypertension, alterations in expression and function of GPCRs play a crucial role. The cellular responses elicited upon activation of GPCRs are tightly regulated by phosphorylation status of the receptors mediated by GRKs. According to the GRK subtypes, phosphorylation can be regulated differentially, leading to distinct modulations of receptor responsiveness or "signalling barcodes" (Butcher *et al.*, 2011). Different phosphorylation sites may provide different conformational changes of the receptor, since the effect of various kinases under identical conditions can result in a different extent of responses (Boughton *et al.*, 2011). In our model, chronic inhibition of GRK2 expression leads to differential increases of the other GRK subtypes (GRK3, GRK4, GRK5, and GRK6) in both aortic and kidney tissue. These increases were synchronized with the onset of the hypertensive state. Whether the activation of these kinases has a distinct role on the

mechanisms of hypertension or their increase engenders a more compensatory role in the attempt to balance the attenuation in GRK2-mediated receptor desensitisation, remains to be determined.

Our data indicates that the blockade of the AT_1 receptor was associated with a significant downregulation in the level of GRK5 mRNA expression. Hypertension itself has been shown to trigger the increase of GRK5 expression and Ca^{++} influx was a prerequisite for this upregulation (Ishizaka *et al.*, 1997). Indeed, our results indicate that the blockade of AT_1 receptor has normalized blood pressure that consequently led to GRK5 decreases, underscoring its counterbalancing role with respect to decreased GRK2-mediated phosphorylation. On the other hand, the amount of GRK3 and GRK4 expressions in response to AT_1R blocker-losartan remained unchanged even though the increase in their levels follows the development of high blood pressure in shGRK2 mice. This might imply that a distinct mechanism other than hypertension was the underlying factor for their upregulation.

Of note, GRK4 has been shown to phosphorylate and desensitize dopamine D_1 receptor and to play a key role in dopaminergic-mediated natriuresis and thereby blood pressure regulation(Yatabe *et al.*, 2008). It seems that GRK2 inhibition can lead to a possible disruption of D_1R gene in the kidney of shGRK2 mice since a significant decrease in D_1 receptor mRNA expression level was measured across the entire lifespan. In addition, the extent of D_1R expression can be correlated to the development of hypertension since its expression was significantly decreased in the pre-hypertensive state, but further dropped after the onset of hypertension. The modulation of D_1 receptor expression may be in part explained through the activation of renal GRK4 since D_1R is a better substrate for GRK4

than GRK2 or GRK3. Thus, the increase in GRK4 expression observed in our shGRK2 transgenic mice can lead to an increase in desensitization of D_1 receptors which in turn has translated into a more pronounced antinatriuretic effect. Indeed, our results indicate that D_1R expression was associated with a decrease in urinary sodium excretion that was markedly attenuated with the development of hypertension. As noted previously, desensitization of D_1R due to GRK4 associates with enhanced expression and activity of AT_1R in the proximal tubules leading to enhanced antinatriuretic effects (Jose *et al.*, 2010; Rayner and Ramesar, 2015).

Past works define the role of AT_1 receptor in renal function and structure through the modulation of glomerular filtration rate, facilitation of renal sodium retention and regulation of cellular growth and differentiation. On the other hand, renal AT_1 receptors have unique and non-redundant actions in blood pressure homeostasis since blood pressure can be regulated by direct effects of AT_1 receptors on kidney cells (Crowley *et al.*, 2006; Wu *et al.*, 2015). In line with these observations, our data indicate that the mRNA expression of the AT_1 receptor was augmented in both aortic and kidney tissue of shGRK2 mice and this increase positively correlates with the development of high blood pressure. Treatment with a specific AT_1R-blocker Losartan has significantly decreased the blood pressure elevation that was brought down to the level of wild type mice. The reduction in blood pressure was a direct consequence of interrupting the AT_1 receptor signaling and this was supported by our results showing that high blood pressure was completely reversed after a three-week washout period.

The role of intra-renal RAS has emerged as pivotal in the management of blood pressure regulation and electrolyte balances (Coffman and Crowley, 2008; Bader, 2010;

Crowley and Coffman, 2012). Intra-renal angiotensin increases during the development of hypertension and renal damage. The overexpression of angiotensinogen in the kidney could lead to chronic hypertension and mice lacking the intra-renal angiotensinogen synthesis are characterized by a lesser extent of hypertensive damage (Crowley *et al.*, 2005; Susic and Frohlich, 2011). We observed that the mRNA expression level of angiotensinogen was augmented in kidneys of shGRK2 mice mainly due to the activation of intra-renal Ang II/ AT_1R axis and this "feed-forward" system may alter sodium reabsorption and consequently blood pressure. The control of renal sodium is the final conduit for chronic blood pressure homeostasis. Previous studies have indicated that AT_1 receptors in the kidney are primary responsible for sodium homeostasis. AT_1 receptors expressed in the proximal tubule promote sodium reabsorption by controlling the Na-K ATPases. In the distal tubule and collecting duct AT_1 receptors directly stimulates epithelial sodium channel activation and promote sodium reabsorption (Harrison-Bernard and Carmines, 1994; Kwon *et al.*, 2003). In addition, renal vasoconstriction mediated by AT_1 receptors can affect the modulatory flow by blunting the kidney excretory function for sodium (Crowley *et al.*, 2006). In the present study, GRK2 downregulation was associated with an enhance in AT_1R signaling and reduced renal sodium excretion. Moreover, the reduced urinary sodium excretion parallels the development of high blood pressure in shGRK2 mice. Indeed, the urinary sodium excretion was reduced by 60% in shGRK2 hypertensive mice, supporting the observation that the control of renal sodium is an important pathway of blood pressure homeostasis.

Renin is a rate-limiting enzyme and its expression/secretion is regulated at the juxta-glomerular apparatus by renal baroreceptors and sodium chloride concentration at

the macula densa (Lorenz *et al.*, 1991; Bell *et al.*, 2003). Our findings indicate that the intra-renal renin protein and mRNA levels increase during the development of hypertension and renal damage in shGRK2 mice. No significant changes were recorded for plasma renin in the pre-hypertensive and three-month old hypertensive mice. However, the level of plasma renin emerged a significant increase in shGRK2 mice at six months of age. Thus, this data emphasizes the role of local RAS in the management of blood pressure. The availability of renin affects the generation of Ang II that may control its own synthesis by activating AT_1R at the juxta-glomerular apparatus and thereby supressing the renin release. The effect of AT_1R blockers to increase the renin mRNA expression and to cause juxta-glomerular hypertrophy supports the "short-loop" feedback mechanisms (Shricker *et al.*, 1997; Oliverio *et al.*, 1998). Our data suggests that the treatment with selective AT_1R-blocker losartan has increased the renal renin mRNA expression more than tenfold above baseline in shGRK2 mice when compared to water controls. The renal baroreceptors mechanisms seem to be responsible for the marked stimulation of the renin associated with the reduction of the AT_1R signaling (Bock *et al.*, 1992). Indeed, our findings indicate an average reduction of 4 0 mm Hg in blood pressure of shGRK2 mice in response to losartan that could trigger the baroreceptor mechanisms to activate the increase in renal renin expression.

Along with its enzymatic properties, renin – through the activation of pro-renin receptors in kidney glomerulus – stimulates pro-fibrotic and inflammatory pathways (Balakumar and Jagadeesh, 2010; Nguyen and Muller, 2010). Moreover, one of the direct effects of AT_1 receptor activation is end-organ injury by inducing the expression of pro-inflammatory cytokines and fibrosis markers (Crowley *et al.*, 2008). We found that the

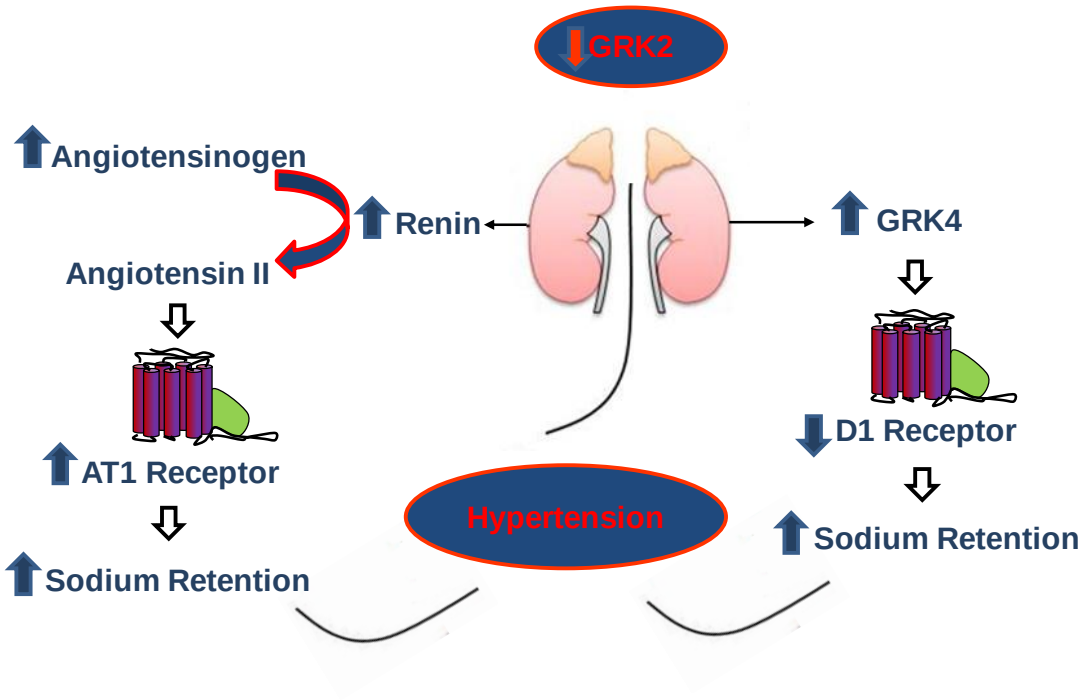
chronic activation of AT_1 receptor signaling due to reduced receptor desensitization is associated with elevated levels of $TGF\beta_1$ and collagen expression and thereby reflects the effects on renal fibrosis observed in our shGRK2 mice. $TGF\beta_1$ is a key regulator of renal fibrosis through the regulation of the extracellular matrix surrounding the renal cells (Kagami *et al.*, 1994). Thus, we found an increased collagen deposition, especially around the renal arteries, with a significant reduction of the glomerular number.

The extensive loss of GRK2 expression through the activation of $AngII/AT_1$ receptor axis not only promotes the renal pathological injuries but also the functional ones. A significant impairment in glomerular filtration rates was noticed for GRK2 hypertensive mice. The treatment with the ARB losartan markedly improved GFR indicating that the activation of Ang II/AT_1R axis could be responsible for renal functional injury. Our findings also indicate low serum albumin levels for three-month old shGRK2 mice, age that corresponds to the onset of hypertension. This pattern of expression was still maintained at six months of age. In addition, the presence of proteinuria and the increase in blood urea nitrogen in shGRK2 mice are direct reflections of the functional damage of the kidney.

In summary, the kidney injury described in our model may promote and maintain the hypertensive phenotype observed in shGRK2 mice and therefore emphasize the role of target organ injury in the management of hypertension. The chronic activation of RAS not only leads to defective renal-sodium excretion but also potentiates renal injury and thereby could be responsible – at least in part – for the hypertensive phenotype. In addition, the effect of GRK4 activation on dopaminergic-mediated natriuresis potentiates and further sustains the alteration of kidney excretory function for sodium (Fig. 3.8).

Figure 3.8: Schematic diagram of the proposed role of GRK2-targeted knockdown expression in the mechanism of hypertension

Inhibition of GRK2 activity leads to chronic activation of AT_1 receptor signaling due to reduced receptor desensitization. In addition, the increased expression of renin and angiotensinogen levels modulates the activation of Ang II / AT_1 receptor axis with a strong impact on the regulation of urinary sodium excretion. As the blood pressure increases, the chronic activation of RAS not only leads to defective renal-sodium excretion, but also potentiates renal injury by inducing alterations in glomerular filtration rates and progression of renal fibrosis. In addition, the up-regulation of GRK4 expression leads to an increase in desensitization of D_1 receptors, which in turn translates into a more pronounced anti-natriuretic effect. Thereby, this “feed-forward” system could be deemed responsible – at least in part – for the hypertensive phenotype.


GRK2
Angiotensinogen
Renin
GRK4
Angiotensin II
D1 Receptor
AT1 Receptor
Hypertension
Sodium Retention
Sodium Retention

Regardless of their initiating factor, decreases in sodium excretory capabilities lead to elevation of blood pressure and this supports the observation that the control of renal sodium is an important pathway of blood pressure homeostasis.

3.5 References

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CHAPTER 4:

MODULATION OF RAB4-GTPase ACTIVITY RESULTS IN ALTERED VASCULAR GPCRs SIGNALING AND HYPERTENSION

4.1 Introduction

The amplitude of cellular responses depends in part on the number of functional receptors available at the cell surface (Lefkowitz *et al.*, 1998; Moore *et al.*, 2007). GPCR expression on the cell surface represents a dynamic, but coordinated balance between molecular mechanisms governing: receptor internalization, endocytosis, degradation, and recycling. GPCR internalization, initiated within seconds to minutes upon receptor activation, is one mechanism by which cells can terminate cellular responses (Ferguson and Caron, 1998; Krupnick and Benovic, 1998; Ferguson, 2001). To prevent the prolonged deactivation, the internalized receptor are recycled back to the plasma membrane to restore the complement of cell surface receptors and functional signaling (Anborgh *et al.*, 2000; Li *et al.*, 2000; Seachrist *et al.*, 2002; Marchese *et al.*, 2008).

The vesicular transport of GPCRs through functionally distinct intracellular compartments is regulated temporally and spatially by Rab GTPases. By serving as a scaffold for binding unique effectors, Rab proteins regulate discrete and multiple steps in endocytosis including: vesicular membrane budding, trafficking along the cytoskeleton, and ultimately docking and fusion between donor and acceptor membranes (McLauchlan *et al.*, 1998; Gonzalez and Scheller, 1999; Mohrmann and van der Sluijs, 1999; Nielsen *et al.*, 1999). Rab GTPases are ubiquitously expressed, highly conserved in their structure, and are regulated by GTP binding and hydrolysis as well as by effector protein interactions. Given the low similarity (< 55% identical), a single activated Rab protein can selectively bind to a multitude of effector proteins to coordinate distinct vesicular transport steps (Takai *et al.*, 2001; Rosenfeld *et al.*, 2002; Seachrist and Ferguson, 2003).

Rab4 modulates the fast recycling of cargo proteins directly from the early endosomes to the plasma membrane. Emerging data indicate that Rab4 exhibits overlapping distribution with Rab11 and also controls the slow recycling of cargo proteins via fusion with Rab11-positive recycling endosomes (Sonnichsen *et al.*, 2000; Zerial and McBride, 2001; Seachrist and Ferguson, 2003; Duvernay *et al.*, 2005). Rab4 influence the intracellular trafficking and recycling of several GPCRs, including AT_1R and β_2AR with main role in vascular tone and reactivity (Seachrist *et al.*, 2000; Odley *et al.*, 2004; Li *et al.*, 2008). The internalized AT_1R through the last 10 amino acid residues of carboxyl-terminal tail domain associates in a dynamic manner with Rab4 proteins. Therefore, at the early recycling stage, AT_1R are observed in Rab4 positive compartments in the cytoplasm, while during middle-recycling stage they are present in the perinuclear compartments in association with both Rab4 and Rab11. Finally, in the late recycling stage AT_1R-Rab4 complexes are present in both perinuclear compartments and the subplasma membrane area (Seachrist *et al.*, 2000; Li *et al.*, 2008). The overexpression of either wild-type or constitutively active Rab4-Q67L mutant promotes AT_1R dephosphorylation and leads to increased receptor re-sensitization (Esseltine *et al.*, 2011).

Rab4 plays a central role in regulating the functional activity of the β_2A receptors by mediating the fast recycling route of internalized receptors directly from early endosomes to the plasma membrane (Seachrist *et al.*, 2000). Rab4 is a rate-limiting factor for the recycling of endogenous β_2AR by controlling the rate at which the receptor is processed through the endosomal compartment. The dominant-negative Rab4-N121I mutant blocks β_2AR recycling and re-sensitization while the overexpression of wild-type Rab4 neither alters the apparent rate of β_2AR internalization nor accelerates the recycling

of the receptor back to the cell surface (Seachrist *et al.*, 2000). The expression of constitutively active Rab4-Q72L accelerates β_2AR reinsertion into plasma membranes but had no effects on cardiac structure or function (Odley *et al.*, 2004). On the other hand, the dominant-inhibitory Rab4-S27N mutant prevents β_2AR recycling, causing receptor accumulation in early endosomes and impaired responsiveness to endogenous and exogenous catecholamine (Odley *et al.*, 2004; Filipeanu *et al.*, 2006). Thereby, Rab4-mediated recycling of β_2AR is necessary for normal cardiac adrenergic responsiveness and re-sensitization after agonist exposure. Previous studies indicated that endogenous Rab4 protein expression is augmented in a cardiomyopathy model of heart failure and the overexpression of Rab4 in transgenic mouse hearts leads to alter β_2A receptor re-sensitization and cardiac hypertrophy (Wu *et al.*, 2001; Odley *et al.*, 2004; Filipeanu *et al.*, 2006). Thus, alterations in Rab4 expression and activity have the potential to influence β_2AR signaling under both physiological and pathophysiological conditions, suggesting that this protein may represent a viable target in the treatment of the cardiovascular disease. However, the effects of Rab4 GTPase on the regulation of GPCR signaling in vascular smooth muscle cells and hypertension have not been addressed, largely because suitable *in vivo* experimental models do not exist.

In the present chapter, the main premise of the study was to test whether the transgenic vascular expression of a dominant negative Rab4-S22N mutant leads to alterations in vascular reactivity, vascular tone, and vascular smooth muscle cells signaling that ultimately leads to vascular hypertension.

4.2 Experimental Procedures

4.2.1 Isolation of Aortic Medial Layer and Primary ASMC Culture

Aortic smooth muscle cells (ASMCs) were prepared via collagenase (2000 U/ml) and elastase (1 mg/ml) (Worthington Biochemical) incubation from freshly isolated thoracic aortas obtained from 6 month-old wild type C57BL/6 mice. The full-length aorta was excised, cut open longitudinally and endothelium was removed by abrasion. The medial layer was then peeled off of the adventitia and subjected to enzymatic digestion. Following isolation, ASMCs were grown in Dulbecco's Modified Eagle Medium with 4.5 g/l glucose supplemented with 10% FBS, 50 μg/ml PDGF-B, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO2 at 37ºC. Cells were maintained in cultures for a number of seven passages. To address possible passage-specific changes in ASMCs responses early and late passages were also used. ASMCs were characterized by immunostaining and immunoblotting for smooth muscle-specific α-actin expression.

4.2.2 Adenoviral Infection

cDNA encoding GFP-Rab4, GFP-Rab4-N121I, and a control cDNA encoding only GFP were used to generate adenoviral constructs (AdMax) as per the manufacturer's instructions (Microbix Biosystems, Toronto, ON). All GFP-Rab4 DNA constructs were designed as previously reported (Seachrist *et al.*, 2000). Mouse ASMCs cultured in 60 mm dishes to near confluence (80%) were subjected to infection with viral titers -100 MOI- of the above mentioned adenoviral constructs. Post-infection (48 hours) the efficiency of transduction was confirmed by immunoblot and confocal microscopy for GFP expression.

4.2.3 Measurement of Cyclic Adenosine Monophosphate (cAMP)

ASMCs 48 hours post-infection were re-cultured on 96-well plate at a density of 1 x 10^4 cells/well and the dose response curves for isoproterenol-stimulated cAMP formation (100 pM to 10 μM) were measured by means of a cAMP GLO assay (Promega, Fitchburg, WI, USA) as described previously (Tutunea-Fatan *et al.*, 2015).

4.2.4 Fura - 2 Florescence Ca^{2+} Imaging

Control-GFP, GFP-Rab4 and -Rab4-N121I expressing ASMCs were grown on 25 mm round glass cover ships and loaded with 5μM Fura-2 acetoxymethyl ester (Invitrogen, Mississauga, ON, Canada) for 20 minutes. Intra-cellular Ca^{2+} release in response to stimulation with either AngII (100nM) or Serotonin/5-HT (100 μM) was measured as described previously (Tutunea-Fatan *et al.*, 2015).

4.2.5 Western Blot Analysis

ASMCs were cultured on six-well plates at a density of 2 × 10^5 cells/well and infected for 48 hours. Then, cells were starved for 24 hours and stimulated with isoproterenol (10 μM), carvedilol (10 μM), 5-HT (100 μM), AngII (100nM), or [Sar1, Ile4, Ile8] AngII (100 μM) for various time points. The reactions were terminated by the addition of 80 μl/well of M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA). Fifteen micrograms of total protein were electrophoresed per well on a 10% SDS-polyacrylamide gel and transferred onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA, USA). The membranes were probed with p44/p42 rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) (1:500), phospho-p44/p42 rabbit polyclonal antibody (Cell Signaling Technology) (1:500), phospho-Retinoblastoma

(Ser807/811) rabbit polyclonal antibody (Millipore) (1:1000), and GAPDH mouse polyclonal antibody (Millipore) (1:500). Blots were probed afterwards with IRDye polyclonal secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) (1:10000). The signal was detected using an Odyssey infrared imaging system and the average density of each band was quantified using the Image Studio software (LI-COR Biosciences, Lincoln, Nebraska USA). Data were normalized to GAPDH or to corresponding total protein values, and expressed as percentage over results obtained for untreated conditions.

4.2.6 Functional Assays

ASMCs migration was assessed with Boyden chambers using Transwell® inserts with 8-µm pore size (Corning Life Sciences, Oneonta, NY, USA). Control or adeno-infected cells were seeded at 2×10^5 cells/mL into the upper chambers. ASMCS migration was stimulated with isoproterenol (10 µM) or AngII (100nM), added to the lower chamber of the inserts. After 24-hour incubation, the transwell membrane were fixed in cold methanol, stained with eosin-thiazine and fixed with mounting medium on a glass slide for microscopy analysis. Digital microscopy images at 40 × magnification (Leica DFC 295, Leica Microsystems, Germany) were taken and cells in ten random high power fields were counted and a mean value for each sample was calculated.

ASMCs proliferation was assessed with a Trypan Blue exclusion assay. ASMCs 48-hour post-infection were cultured on six-well plates at a density of 2×10^5 cells/well. After 48 and 72 hours, cells were harvested and suspended with Trypan Blue (1:1). Total cell number, live cells, dead cells, and viability were calculated with an automated cell counter (Invitrogen).

4.2.7 Generation of Rab4-S22N Transgenic Mice

Transgenic mice expressing the Rab4-S22N inhibitory non-GTP binding construct were generated under the control of a murine smooth muscle 22α (SM22α) promoter. The original pUC57-SM22α construct (Gene Script, lot # 136526S) was subjected to a real-time PCR protocol for the amplification of the restriction enzyme sites with the following primers: forward-AseI (5'-AAATTATTGGATTCAGGACGTAATCAGTGG-3') and reverse-NheI (3'-AAGCTAGCGGCTTGGTCGTTTGTGGACTGG-5). Therefore, a final linear SM22α construct of 1.4 Kb was obtained after enzymatic digestion. The resulting SM22α fragment was sub-cloned into the pEGFP-Rab4-S22N-C2 construct (4.9 Kb) digested with *Ase*I and *Nhe*I (Thermo Scientific) and subjected to sequencing for integrity (Fig. 4.5A). Then, the final circular construct (6.3Kb) was digested with ApalI/MluI (Thermo Scientific) to linearize the DNA for microinjection and to remove as much as possible vector sequences from the insert (Fig. 4.5B). The gene construct obtained after backbone removal (3.6 Kb) was microinjected into the pro-nuclei of isogenic C57Bl/6J single-celled oocytes using standard techniques (Transgenic Core Facility, University of Michigan, USA). The putative transgenic mice were identified by real time-PCR analyses of tail DNA for EGFP expression (Fig. 4.5C and 4.5D) to established homozygous mouse colonies after ten generations (F10) of breeding.

4.2.8 DNA Isolation and Real-Time Polymerase Chain Reaction

DNA was extracted from mouse tail biopsies (3mm) by digestion with lysis buffer (5 mM EDTA, 200 mM NaCl, 100 mM Tris, 0.2% SDS) supplemented with protein kinase K (0.4 mg/ml) (Sigma-Aldrich, Oakville, ON, Canada). After incubation at 55°C overnight, follow by several centrifugation steps, DNA was finally eluted with 1xTE buffer (10 mM

Tris-0.2 mM EDTA). cDNA was amplified by real-time PCR with Taq Polymerase (Qiagen, Valencia, MD, and USA). A PCR reaction volume of 25 µl was prepared for amplification with the following primer pair: EGFP, forward 5'-CGA AGG CTA CGT CCA GGA GCG CAC-3', and reverse 3'-GAC ATG GAT CTG AGT CCG GAC-5', 300 bp-as design from the sequence of our DNA transgenic construct (Primer3Plus software). PCR cycling conditions consist of initial denaturation at 94°C for 3 minute and then 28 cycles of denaturation at 94°C (30 seconds), annealing at 60°C (45 seconds), and extension at 75°C (45 seconds). Real-time PCR products were separated by electrophoresis (85V for 50 minutes) on 1.5% agarose gel and visualized by GelRed Nucleic Acid Gel Stain using a gel imaging system (GelDoc^{TM} XR System, Bio Rad).

4.2.9 Analysis of Copy Number Variation

Quantitative real-time PCR was performed in single micro capillary tubes with TaqMan Universal PCR Master Mix (Roche Diagnostic, Laval, QC, Canada) for both the control-housekeeping gene (Pre-Developed Assay Reagent, Mm ACTB) and target gene expression primer probe (Custom Design Assay Reagent, Mm-PN4331348 EGFP, Applied Biosystems, Foster City, CA, USA). The PCR profile consist of denaturation at 95°C for 15 seconds, annealing-extension at 58°C for 1 minute for 40 cycles. Delta-delta Ct (ΔΔCt) method was employed to determine the fold difference ($2^{-\Delta\Delta Ct}$) (Applied Biosystems).

4.2.10 Blood Pressure Measurement

Blood pressures and heart rates were recorded from conscious and lightly anesthetized (Ketamine: 100 mg/kg body weight and Xylazine 5 mg/kg body weight) Rab4-S22N C57BL/6 transgenic mice at 4 weeks, 12 weeks, and 6 months of age using the non-invasive CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA) as

described previously (Tutunea-Fatan *et al.*, 2015). Third, forth , and fifth generation of adult animals (1-6 months of age) were used for this study. All experiments involving animals were approved by the Animal Use Subcommittee of Western University, according to the guidelines of the Canadian Council on Animal Care.

4.2.11 Statistical Analysis

Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA, USA). All parametric data were analyzed with one-way ANOVA followed by Bonferroni post hoc comparisons. Statistically relevant differences between mean values were determined based on $p < 0.05$ criterion.

4.3 Results

4.3.1 Effects of Rab4 Modulation on Heterotrimeric G Protein Signaling

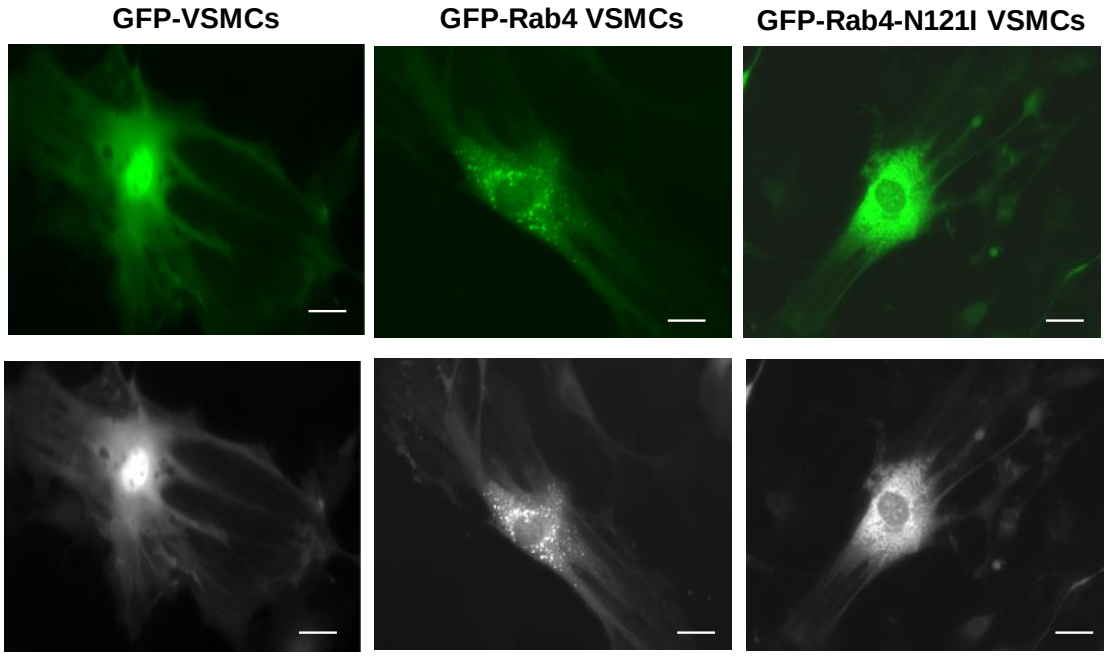
To assess the role of Rab4 *in vitro*, aortic smooth muscle cells obtained by enzymatic digestion from 6 month-old C57Bl/6 mice were subjected to adenoviral infection with the following constructs: control GFP, GFP-Rab4, or dominant negative mutant, GFP-Rab4N121I. The efficiency of transduction was assessed at the protein level for both GFP and Rab4 expressions (Fig. 4.1A and 4.1B).

It is noteworthy that, the adenoviral expression of Rab4 constructs produced different effects on mRNA expression level of vascular GPCRs (Fig. 4.2A). Whereas mRNA expression of β_2AR was not altered by wild type Rab4, adenoviral expression of Rab1N124I inhibitory construct significantly augmented the level of its expression when compared with control GFP-VSMCs. The expression level of AT_1 receptor was significantly increased in the presence of Rab4 construct while the dominant negative

Figure 4.1: Assessment of adenoviral infection

(A) Representative confocal microscopy images of GFP expression in VSMCs 48 h post-infection. GFP tagged Rab4 and Rab4-N121I constructs are depicted in the form of vesicular structures distributed all over the cytoplasm. GFP alone was used as a control. Images were taken under 20X magnification **(B)** Western blot analysis of Rab4 and GFP protein expression detected at 53kDa in VSMCs. GAPDH was used as a loading control.

A)


GFP-VSMCs
GFP-Rab4 VSMCs
GFP-Rab4-N121I VSMCs

B)

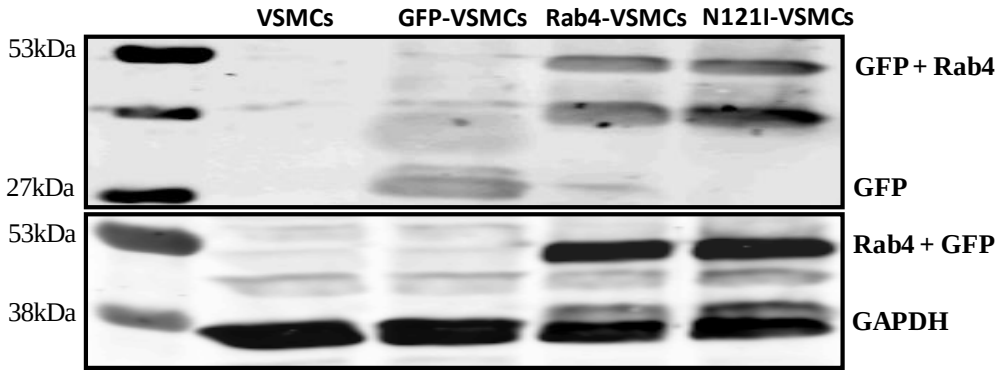
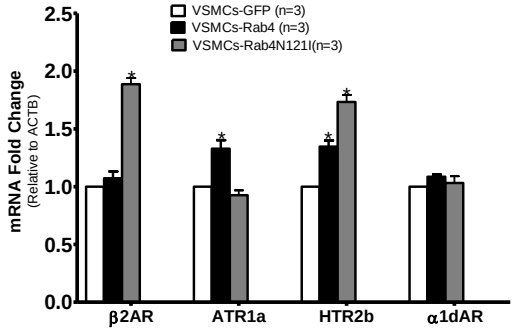

VSMCs
GFP-VSMCs
Rab4-VSMCs
N121I-VSMCs
53kDa
27kDa
GFP + Rab4
GFP
53kDa
38kDa
Rab4 + GFP
GAPDH

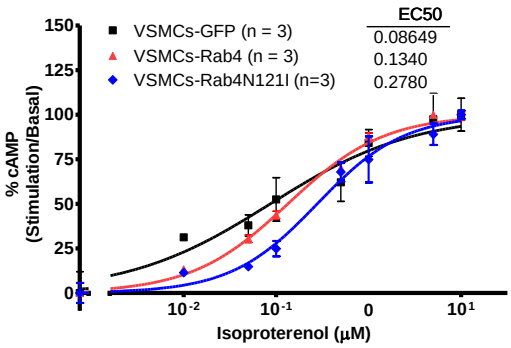
Figure 4.2: Assessment of heterotrimeric G protein signaling

(A) Quantitative PCR analysis of vascular GPCRs mRNA expression in VSMCs subjected to: GFP, Rab4, and Rab4N121I adenoinfection. Expression levels are normalized to actin (ACTB). **(B)** Dose response curves for cAMP production in response to treatment with increasing concentrations of isoproterenol. Data points are represented as the mean ± SD of 3 independent experiments. **(C)** Representative Ca^{2+} tracers for changes in intracellular Ca^{2+} concentration in VSMCs cultures 48 h post-infection in response to treatment with AngII and 5-HT. Graph shows the area under the curve (AUC) for integrated Ca^{2+} responses to agonist treatments. Data are represented as the mean ± SD of 4 independent experiments and the total number of cells analyzed is shown in the graph.

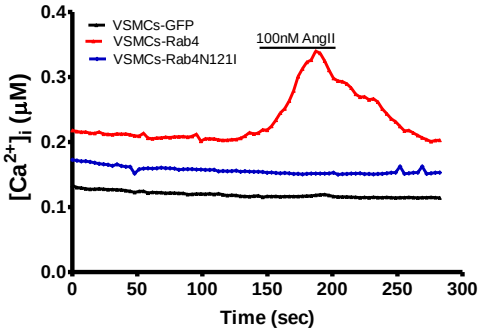
A)

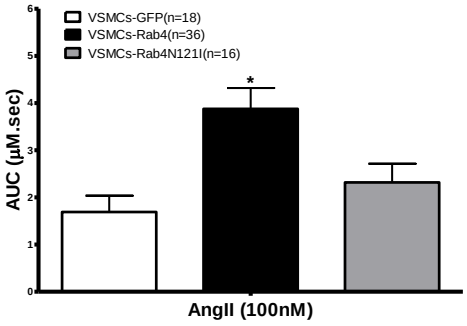

VSMCs-GFP (n=3)
VSMCs-Rab4 (n=3)
VSMCs-Rab4N121I(n=3)
mRNA Fold Change
(Relative to ACTB)
2.5
2.0
1.5
1.0
0.5
0.0
β2AR
ATR1a
HTR2b
α1dAR

B)

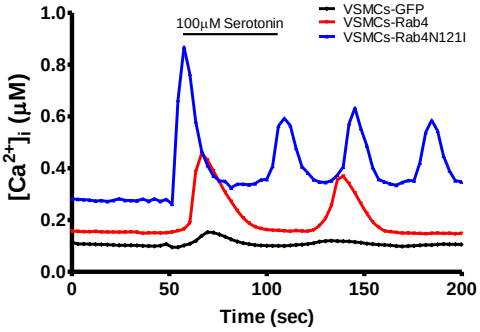

EC50
VSMCs-GFP (n = 3)
0.08649
VSMCs-Rab4 (n = 3)
0.1340
VSMCs-Rab4N121I (n=3)
0.2780
% cAMP
(Stimulation/Basal)
150
125
100
75
50
25
0
10-2
10-1
0
101
Isoproterenol (μM)

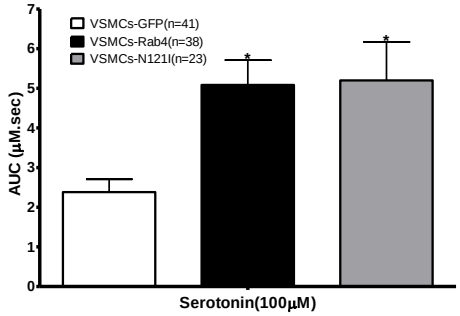
C)


VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I
100nM AngII
[Ca2+]i (μM)
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
250
300
Time (sec)


VSMCs-GFP(n=18)
VSMCs-Rab4(n=36)
VSMCs-Rab4N121I(n=16)
AUC (μM.sec)
AngII (100nM)

D)


100μM Serotonin
VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I
[Ca2+]i (μM)
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Time (sec)


VSMCs-GFP(n=41)
VSMCs-Rab4(n=38)
VSMCs-N121I(n=23)
AUC (μM.sec)
Serotonin(100μM)

Rab1N124I mutant had no effect when compared with cells infected with adenovirus control. On the other hand, the mRNA expression of serotonin-HTR2b-receptor was significantly increased in cells subjected to both Rab4 and Rab4N121I constructs. No effects were noticed for α_{1d}-AR receptor regardless if cells were infected with Rab4 or Rab4N121I mutant.

To determine whether the manipulation of Rab4 expression and activity could modulate β_2AR signaling, cyclic adenosine monophosphate (cAMP) accumulation in response to the β_2AR agonist-isoproterenol was assessed. The overexpression of wild type Rab4 did not alter the apparent rate of cAMP production. The expression of dominant inhibitory Rab4N121I markedly impaired β_2A receptor responsiveness and consequently cAMP production when compared with control GFP expressing cells (EC_{50}:0.27 vs. EC_{50}:0.08) (Fig. 4.2B). Thus, a loss of Rab4 activity appears to lead to decreased of $G\alpha_s$-mediated receptor signaling.

The release of Ca^{2+} from intracellular stores in response to treatment with 100 nM Ang II elicited a significantly greater increase in VSMCs overexpressing the Rab4 construct when compared to control cells. Expression of dominant inhibitor Rab4N121I had no effects on Ca^{2+} concentration in response to stimulation with AngII (Fig. 4.2C). On the other hand, treatment with serotonin significantly augmented the release of Ca^{2+} whether cells were infected with Rab4 or Rab4N121I mutant (Fig. 4.2D). These results indicate that Rab4 expression not only played a significant role in regulating GPCR expression in VSMCs but receptor signaling is also significantly altered.

4.3.2 Effects of Rab4 Modulation on G Protein-Dependent and Independent ERK1/2 Signaling

To determine whether Rab4 is capable of regulating β_2AR signaling, ERK1/2 activation in response to stimulation with β_2AR agonist-isoproterenol was evaluated by measuring its level of phosphorylation. While the presence of ERK1/2 phosphorylation was detected in VSMCs overexpressing Rab4, the extent of phosphorylation status was unchanged regardless the duration of treatment. In addition, the phosphorylation signal observed for untreated, 48-hour-serum starved cells suggests that ERK1/2 might be constitutively activated in VSMCs overexpressing the Rab4 construct. The depletion of Rab4 significantly increased the phosphorylation status of ERK1/2 when compared with control GFP cells (Fig. 4.3A). Isoproterenol treatment elicited a rapid increase in ERK1/2 phosphorylation with a peak of activation observed at 5 and 15 min of agonist stimulation.

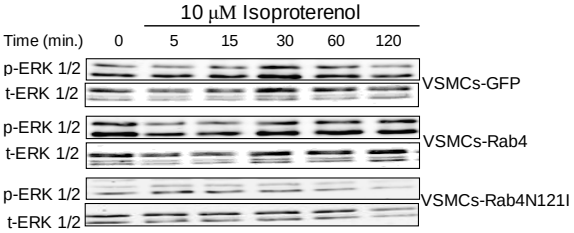
Adenoviral expression of wild type Rab4 augmented ERK1/2 phosphorylation in response to stimulation with β_2AR biased agonist carvedilol. In contrast, carvedilol-mediated ERK1/2 activation was attenuated by Rab4N121I, suggesting that augmentation of Rab4 function by overexpressing Rab4 may selectively regulate the β-arrestin-dependent mechanism (Fig. 4.3B).

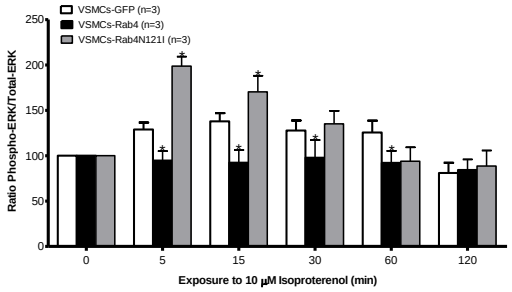
A significant increase in the extent and duration of ERK1/2 activation was observed in response to the treatment of VSMCs expressing the wild type Rab4 with AngII. However, Rab4N121I did not affect the level of Ang II mediated ERK1/2 phosphorylation when compared with control GFP infected cells (Fig. 4.3B).

Figure 4.3: Assessment of $G\alpha_s$-and $G\alpha_q$-mediated Erk1/2 signaling

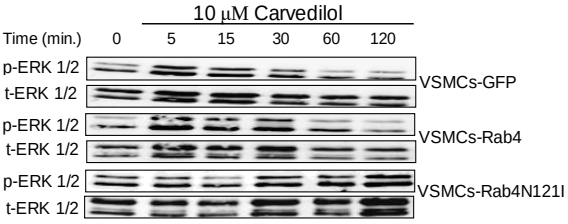
Representative immunoblots and densitometric analysis showing time course for ERK1/2 phosphorylation with corresponding total ERK1/2 immunoblots from VMSCs-GFP, GFP-Rab4, and GFP-Rab4N121I in response to: **(A)** 10 μM isoproterenol, **(B)** 10 μM carvedilol, (**C)**100 nM AngII, (**D)** 100 μM [Sar1, Ile4, Ile8] AngII (SIIAngII), **(E)** 100 μM serotonin. Data are represented as the mean ± SD of 3 independent experiments expressed as percentage of basal ERK1/2 phosphorylation.

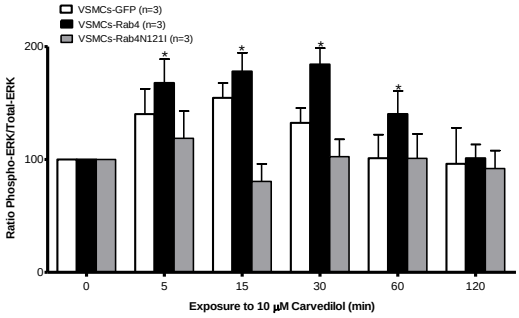
A)


10 μM Isoproterenol
Time (min.) 0 5 15 30 60 120
p-ERK 1/2
t-ERK 1/2
VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I

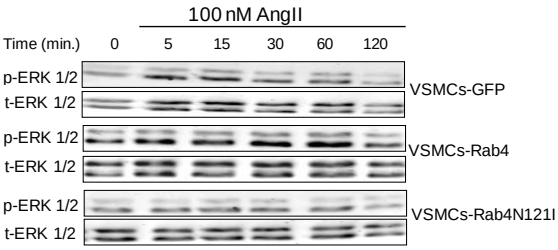

VSMCs-GFP (n=3)
VSMCs-Rab4 (n=3)
VSMCs-Rab4N121I (n=3)
Ratio Phospho-ERK/Total-ERK
Exposure to 10 μM Isoproterenol (min)

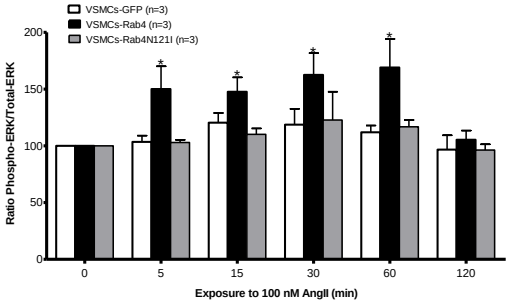
B)


10 μM Carvedilol
Time (min.) 0 5 15 30 60 120
p-ERK 1/2
t-ERK 1/2
VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I

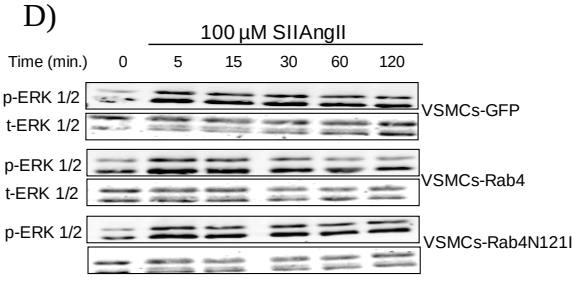

VSMCs-GFP (n=3)
VSMCs-Rab4 (n=3)
VSMCs-Rab4N121I (n=3)
Ratio Phospho-ERK/Total-ERK
Exposure to 10 μM Carvedilol (min)

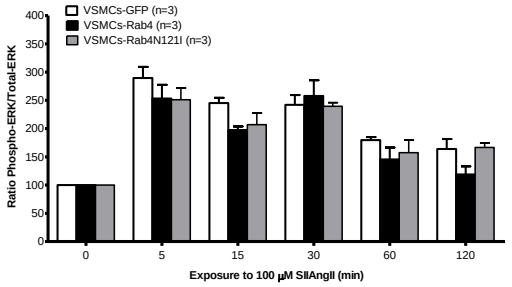
C)


100 nM AngII
Time (min.) 0 5 15 30 60 120
p-ERK 1/2
t-ERK 1/2
VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I

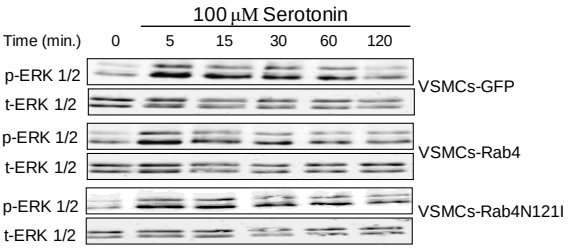

VSMCs-GFP (n=3)
VSMCs-Rab4 (n=3)
VSMCs-Rab4N121I (n=3)
Ratio Phospho-ERK/Total-ERK
Exposure to 100 nM AngII (min)

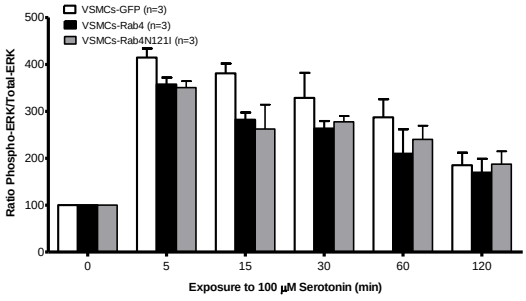
D)


100 μM SIIAngII
Time (min.) 0 5 15 30 60 120
p-ERK 1/2
t-ERK 1/2
VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I


VSMCs-GFP (n=3)
VSMCs-Rab4 (n=3)
VSMCs-Rab4N121I (n=3)
Ratio Phospho-ERK/Total-ERK
Exposure to 100 μM SIIAngII (min)

E)


100 μM Serotonin
Time (min.) 0 5 15 30 60 120
p-ERK 1/2
t-ERK 1/2
VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I


VSMCs-GFP (n=3)
VSMCs-Rab4 (n=3)
VSMCs-Rab4N121I (n=3)
Ratio Phospho-ERK/Total-ERK
Exposure to 100 μM Serotonin (min)

A similar response profile was observed in response to treatment with the biased angiotensin receptor agonist [Sar^1, Ile^4, Ile^8] Ang II whether cells were expressing the wild type Rab4 or dominant negative Rab4N121I (Fig. 4.3C).

Serotonin treatment of either control, wild-type or Rab4 inhibitory VSMCs elicited a rapid increase in ERK1/2 phosphorylation at 5 and 15 min of agonist treatment, activation that was also prolong over the entire duration of stimulation. Thus, the activation of ERK1/2 signaling was unaffected by alteration in Rab4 expression/activity (Fig. 4.3C).

4.3.3 Effects of Rab4 Modulation on VSMC Migration

To assess whether Rab4 modulates long term cellular responses, such as VSMCs migration, a Boyden chamber assay was performed using trans-well inserts. VSMCs subjected to Rab4 expression showed a reduction in cell migration in response to isoproterenol treatment when compared to SFM media. In VSMCs subjected to Rab4N121 inhibitory construct, isoproterenol-stimulated cellular migration was significantly increased when compared with isoproterenol-stimulated migration of VSMCs expressing either the control GFP or Rab4 adenoviral constructs. VSMCs subjected to stimulation with Ang II exhibited a significant increase in migration rate in the presence of Rab4N121I when compared with both control and Rab4 expressing cells (Fig. 4.4A). Thus, Rab4N121I increased VSMCs migration in response to both $G\alpha_s$- and $G\alpha_{q/11}$-coupled GPCR activation.

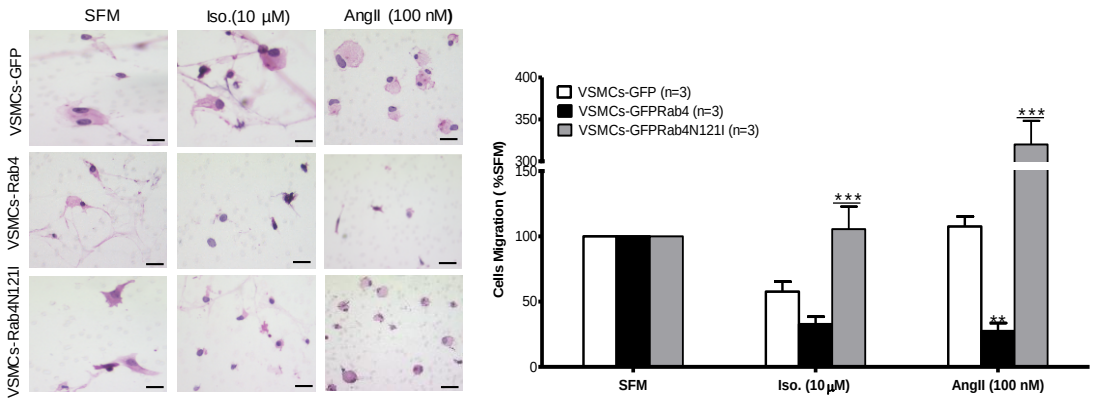
4.3.4 Effects of Rab4 Modulation on VSMC Proliferation

VSMCs expressing Rab4 or Rab4N121I exhibited a significant impairment in the proliferation rate in complete (10% FBS) media when compared with control GFP expressing cells.

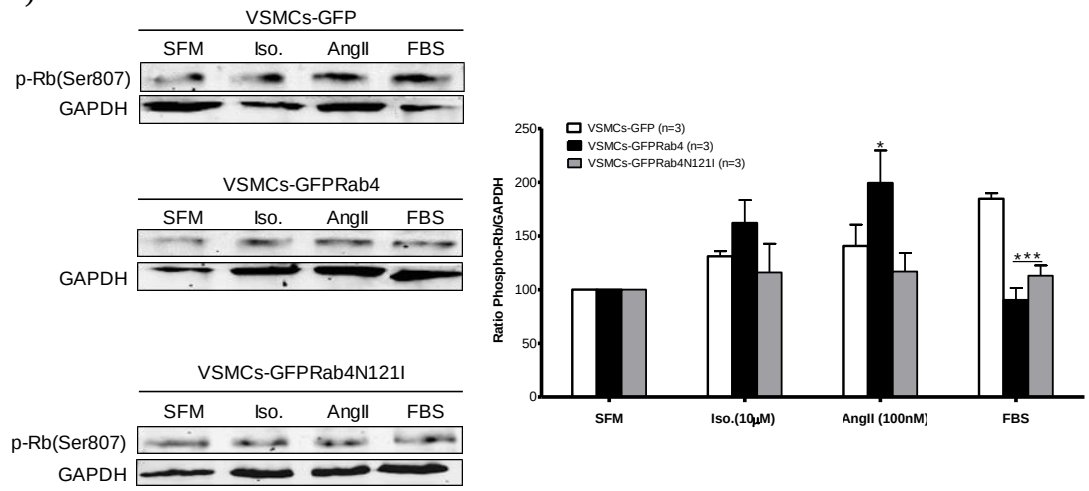
Figure 4.4: Assessment of migration and proliferation responses

(A) Representative images and quantification of the migration of VSMCs adenoinfected with control-GFP, GFP-Rab4, and GFP-Rab4N121I constructs, in response to treatment with 10 μM isoproterenol, or 100 nM Ang II for 24 hours. SFM only was used as a control. Bar graph data represents the mean ± SD of 3 independent experiments expressed as percentage of basal-untreated cells (SFM). **(B)** Representative immunoblots and densitometric analysis showing the phosphorylation status of retinoblastoma protein in response to isoproterenol (10 μM), AngII (100 nM), and serotonin (100 μM), or FBS (10%). GAPDH was used as a loading control. Data are represented as the mean ± SD of 3 independent experiments expressed as percentage of basal-untreated cells (SFM). (**C)** Growth curves over 72-hour course time of primary VSMCs adenoinfected with control-GFP, GFP-Rab4, and GFP-Rab4N121I constructs. Data are represented as the mean ± SD ($n = 3$). (*) Indicates statistically significant differences compared to control ($p < 0.05$).

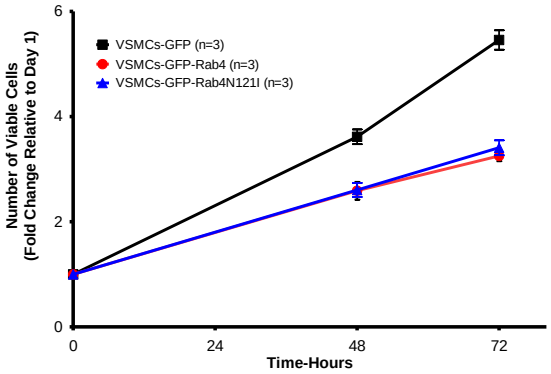
A)


SFM
Iso.(10 μM)
AngII (100 nM)
VSMCs-GFP
VSMCs-Rab4
VSMCs-Rab4N121I
VSMCs-GFP (n=3)
VSMCs-GFPRab4 (n=3)
VSMCs-GFPRab4N121I (n=3)
Cells Migration (%SFM)
400
350
300
150
100
50
0
***
***
**
SFM
Iso. (10 μM)
AngII (100 nM)

B)


VSMCs-GFP
SFM
Iso.
AngII
FBS
p-Rb(Ser807)
GAPDH
VSMCs-GFPRab4
SFM
Iso.
AngII
FBS
GAPDH
VSMCs-GFPRab4N121I
SFM
Iso.
AngII
FBS
p-Rb(Ser807)
GAPDH
VSMCs-GFP (n=3)
VSMCs-GFPRab4 (n=3)
VSMCs-GFPRab4N121I (n=3)
Ratio Phospho-Rb/GAPDH
250
200
150
100
50
0
*
***
SFM
Iso.(10μM)
AngII (100nM)
FBS

C)


VSMCs-GFP (n=3)
VSMCs-GFP-Rab4 (n=3)
VSMCs-GFP-Rab4N121I (n=3)
Number of Viable Cells
(Fold Change Relative to Day 1)
6
4
2
0
0
24
48
72
Time-Hours

Isoproterenol-stimulated VSMCs proliferation was unaffected regardless if cells were infected with Rab4 or Rab4N121I mutant since the extent of retinoblastoma phosphorylation (p-RB) was almost the same. However, Rab4-overexpressingVSMCs subjected to AngII treatment exhibited an increased proliferation rate as indicated by the high phosphorylation level of retinoblastoma protein. On the other hand, Rab4-depleted VSMCs did not significantly alter the phosphorylation status of retinoblastoma protein in response to Ang II treatment then control VSMCs (Fig. 4.4B and 4.4C). Thus, the overexpression of Rab4 wild-type or mutant has proved a role for Rab4 in G protein-mediated long term migration and proliferation of VSMCs responses.

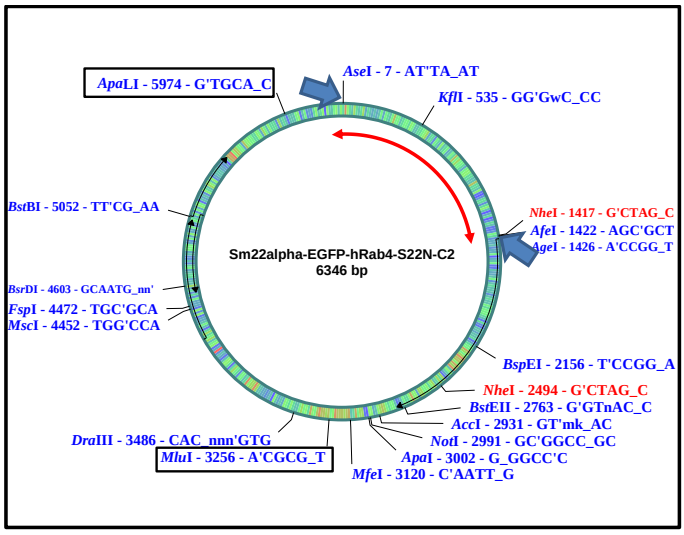
4.3.5 SM22α-Rab4S22N Mice Develop Hypertension with Age

The pathophysiological effects of modulated Rab4 expression/activity in the hypertension have not been defined, largely because suitable *in vivo* experimental models do not exist. To establish the relevance of Rab4 activity in the regulation of the vascular GPCRs signaling *in vivo*, we have used mutation and transgenic expression of a dominant negative Rab4S22N to attenuate endogenous Rab4 function. We generated transgenic mice that specifically overexpress GFP-Rab4-S22N in VSMCs under the control of a murine SM22α promoter (Fig. 4.5). SM22α was used as a promoter since is exclusively expressed in smooth muscle of adult animals and it is one of the markers of differentiated smooth muscle cells. No phenotypic changes or embryonic and neonatal mortality were observed among the SM22α-Rab4S22N transgenic mice when compared with non-transgenic littermate controls. Our observations document a gradual onset of hypertension (Fig. 4.6A).

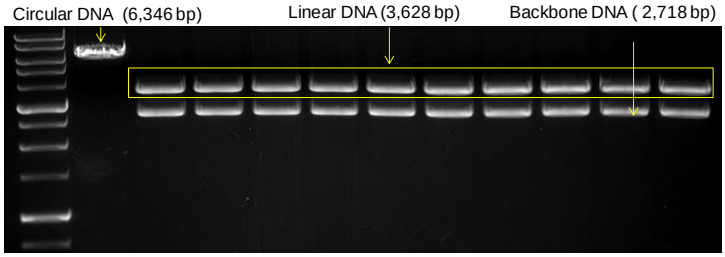
Figure 4.5: Development of the SM22α-Rab4S22N DNA transgene construct and genotyping analysis

(A) Schematic representation of the final 6.3Kb size DNA plasmid construct obtained after sub-cloning the SM22α promoter (1.4Kb) into the pEGFP-Rab4S22N-C2 vector. **(B)** Agarose gel electrophoresis of Sm22alpha-pEGFP-Rab4S22N-C2 linear construct (3.6Kb) after digestion with restriction enzymes ApalI/MluI and backbone removal (2.7Kb) **(C)** Copy number of the Sm22alpha-pEGFP-Rab4S22N-C2 transgene construct spiked into C57Bl/6 tail DNA as measured by Real-time PCR assay. **(D)** Genotyping results as depicted by quantitative and semi-quantitative real-time PCR assays. A custom design EGFP-FAM tagged primer probe was used for quantification of the copy number of the Rab4 transgene. Both, beta globin and ACTB were used as an internal control.

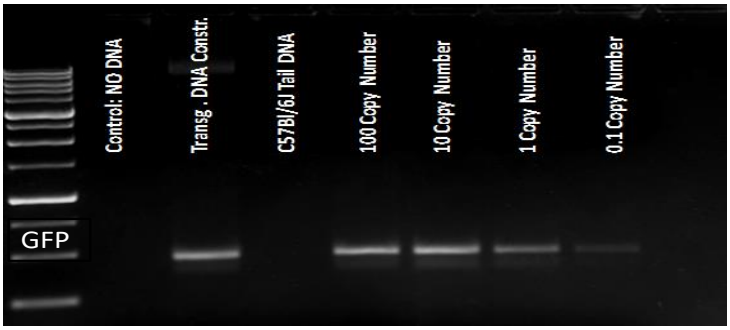
A)


ApaLI - 5974 - G'TGCA_C
AseI - 7 - AT'TA_AT
KflI - 535 - GG'GwC_CC
BstBI - 5052 - TT'CG_AA
NheI - 1417 - G'CTAG_C
AfeI - 1422 - AGC'GCT
AgeI - 1426 - A'CCGG_T
Sm22alpha-EGFP-hRab4-S22N-C2
6346 bp
BsrDI - 4603 - GCAATG_nn'
FspI - 4472 - TGC'GCA
MscI - 4452 - TGG'CCA
BspEI - 2156 - T'CCGG_A
NheI - 2494 - G'CTAG_C
BstEII - 2763 - G'GTnAC_C
AccI - 2931 - GT'mk_AC
NotI - 2991 - GC'GGCC_GC
ApaI - 3002 - G_GGCC'C
MfeI - 3120 - C'AATT_G
DraIII - 3486 - CAC_nnn'GTG
MluI - 3256 - A'CGCG_T

B)


Circular DNA (6,346 bp)
Linear DNA (3,628 bp)
Backbone DNA (2,718 bp)

C)


Control: NO DNA
Transg. DNA Constr.
C57Bl/6J Tail DNA
100 Copy Number
10 Copy Number
1 Copy Number
0.1 Copy Number
GFP

D)

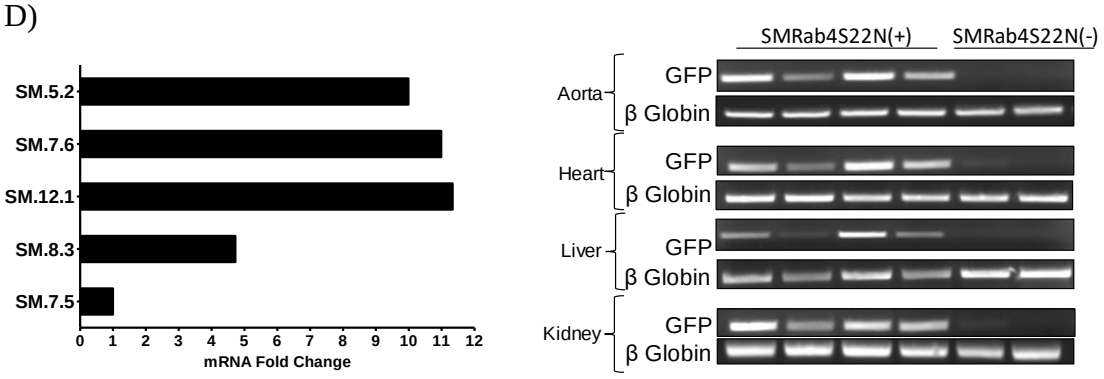
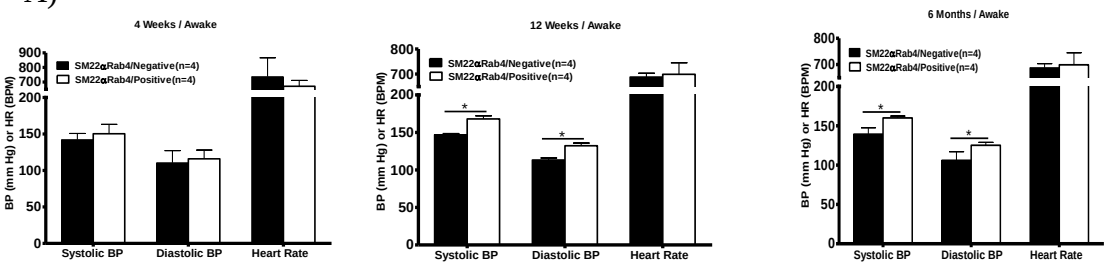

SM.5.2
SM.7.6
SM.12.1
SM.8.3
SM.7.5
0 1 2 3 4 5 6 7 8 9 10 11 12
mRNA Fold Change
SMRab4S22N(+)
SMRab4S22N(-)
Aorta
Heart
Liver
Kidney
GFP
β Globin

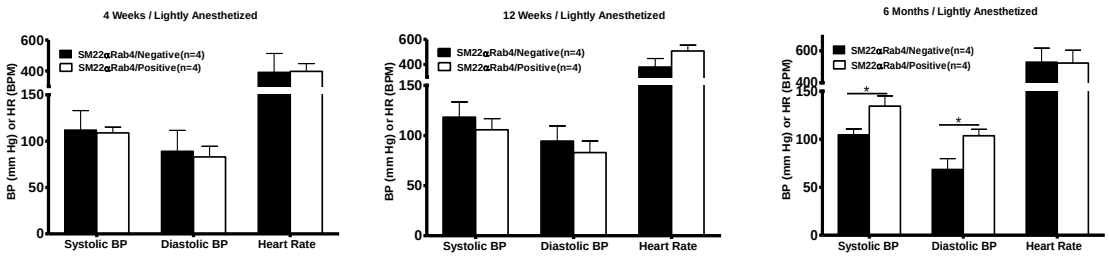
Figure 4.6: Assessment of blood pressure

(A) Blood pressures and heart rates of C57Bl/6-Rab4S22N and control littermate mice, as measured by non-invasive tail cuff CODA method at 4 weeks, 12 weeks, and 6 months of age. **(B)** Blood pressures and heart rates in lightly anesthetized 4 weeks, 12 weeks, and 6 month-old mice using the tail-cuff CODA method. Data are represented as the mean ± SD of 4 independent experiments (n=6). * Indicates statistically significant differences ($p < 0.05$).

A)


4 Weeks / Awake
12 Weeks / Awake
6 Months / Awake
SM22αRab4/Negative(n=4)
SM22αRab4/Positive(n=4)
BP (mm Hg) or HR (BPM)
Systolic BP
Diastolic BP
Heart Rate

B)


4 Weeks / Lightly Anesthetized
12 Weeks / Lightly Anesthetized
6 Months / Lightly Anesthetized
SM22αRab4/Negative(n=4)
SM22αRab4/Positive(n=4)
BP (mm Hg) or HR (BPM)
Systolic BP
Diastolic BP
Heart Rate

Therefore, hypertension was established in 3 month-old SM22α-Rab4S22N mice with a significant increase in both systolic and diastolic blood pressures (168/132 ± 10/6 vs. 142/113 ± 8/11mmHg, $p < 0.05$, n=8). The elevated blood pressures were still maintained at 6 months of age (160/125 ± 9/7 vs. 139/106 ± 7/10 mmHg, $p < 0.05$, n=8). Heart rate was unchanged between the groups. To eliminate the possibility of stress-induced elevations, blood pressure was assessed in anesthetized mice and both systolic and diastolic were increased at 6 months of age when compared with control littermates (135/104 ± 5/7 vs. 105/69 ± 7/6 mmHg, $p < 0.05$, n=8) (Fig. 4.6B).

So far, our preliminary data suggest that the loss of Rab4 activity may favor an increase in peripheral resistance with an increased sensitivity to stress-anxiety leading to the activation of the sympathetic responses. These observations seem to be in agreement with previous work that Rab4-mediated recycling of βAR is necessary for normal cardiac catecholamine responsiveness and receptor re-sensitization after agonist exposure.

4.4 Discussion

Blood pressure homeostasis is tightly regulated by a delicate balance of vasoconstrictive and vasodilatatory signaling inputs and any alterations brought to this "unstable equilibrium" state can trigger a domino effect to tilt the balance in either direction. The most consistently described vascular GPCR-defect in hypertension is the impaired relaxation due to alterations in $G\alpha_s$-mediated receptor signaling (Marcil *et al.*, 1997; Feldman and Gros, 1998; Ferguson and Feldman, 2014). Indeed, a decrease in GPCR signaling can lead to an increased blood pressure via reductions in β_2-adrenergic mediated dilatation.

Cell surface expressions of β_2A receptors linked to vasodilatatory mechanism are preferentially regulated by modulations of Rab4 GTPase activity (Seachrist *et al.*, 2000; Odley *et al.*, 2004; Li *et al.*, 2008). Rab4 is a rate-limiting factor in the regulation of the functional activity of β_2ARs. This is attained through the control of the rate at which the receptor is processed through the endosomal compartments and then recycled back to the plasma membrane. Emerging data indicate that dominant-inhibitory Rab4 mutants prevent β_2AR recycling, causing receptor accumulation in early endosomes. Indeed, confocal studies reveled abnormal vesicular structures and increased accumulation of β_2AR in caveolae and early endosomes (Seachrist *et al.*, 2000; Odley *et al.*, 2004). Aligned with these findings, our data indicate that the expression of a dominant inhibitory Rab4N121I has significantly increased the β_2AR mRNA expression level in VSMCs. The increased gene transcriptional activity of β_2AR may in turn compensate for the impaired recycling of internalized receptors to the plasma membrane. Thereby, Rab4-mediated recycling of the internalized β_2AR plays more of a "housekeeping" role in maintaining the number of functional receptors.

The classical pathway regulating β_2AR signaling involves $G\alpha_s$-mediated activation of adenylyl cyclase and subsequent activation of the cyclic AMP production (Neer, 1995). We found that the transient expression of Rab4N121I in VSMCs has significantly impaired β_2A receptor responsiveness and consequently isoproterenol-stimulated cAMP production, when compared with control GFP expressing cells. Thereby, the low level of cAMP may function as a positive feedback regulator of β_2A receptor gene that ultimately will increase the receptor population.

On the other hand, the overexpression of wild type Rab4 neither alter the apparent rate of cAMP production nor the mRNA expression level of β_2AR in VSMCs. Our results are in alignment with previous studies outlining that an increase in Rab4 expression level did not cause increases in the rates of receptor internalization and re-sensitization (Seachrist *et al.*, 2000). Moreover, cardiomyocyte-specific expression of constitutively active Rab4Q72L had no effect on β_2AR signaling and cardiac function (Odley *et al.*, 2004).

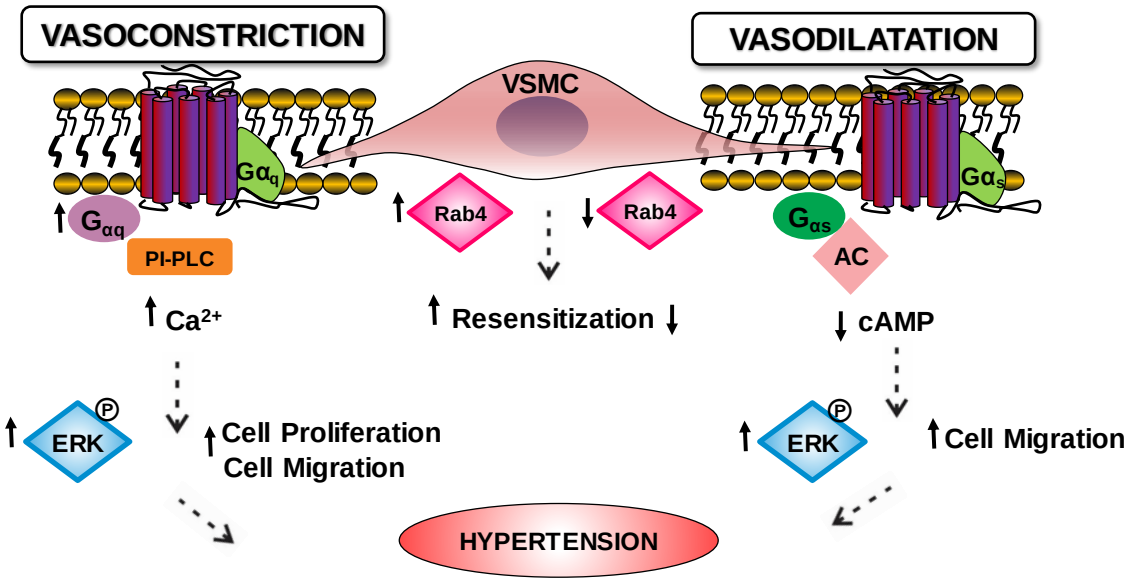
Although AT_1R is not readily dephosphorylated and efficiently recycled (Anborgh *et al.*, 2000; Dale *et al.*, 2004) recent evidences indicate that the overexpression of either wild-type or constitutively active Rab4 in human embryonic (HEK) 293 cell line promotes AT_1R dephosphorylation and recycling back to the plasma membrane (Esseltine *et al.*, 2011). Indeed, our data indicate that while vascular transient expression of wild type Rab4 significantly increases the release of Ca^{2+} from intracellular stores, the expression of inhibitory RabN1221 had no effect on Ca^{2+} concentration in response to AT_1R stimulation. One possible explanation for these different outcomes could be related to the fact that AT_1R seems to not be able to distinguish between GTP-and GDP-bound forms of Rab4 (Esseltine *et al.*, 2011). Additionally, AT_1R trafficking is regulated by Rab4 and Rab11 coordinated model of interaction according to which Rab4 facilitates the early recycling stage, while Rab11 regulates the slow recycling route from perinuclear endosomes to plasma membrane (Sonnichsen *et al.*, 2000; Zerial and McBride, 2001; Seachrist and Ferguson, 2003; Duvernay *et al.*, 2005). Therefore – as AT_1R internalizes as a complex with β-arrestin and is retained in the endosomal compartment in a non-dephosphorylated state – AT_1R might be preferentially recycled via the slow Rab11-mediated pathway. This could explain why

Rab4 inhibition has no effect on AT_1R levels and activity. Thus, taken together these results indicate that alterations in Rab4 expression and activity might differently regulate the recycling of β_2AR and AT_1R to the plasma membrane as well as their subsequent functional cellular responses.

An increased body of work has showed that Rab4 regulates endothelial cell migration, proliferation, and angiogenesis (Jopling *et al.*, 2014; Talaber *et al.*, 2014). In addition, Rab4 performs an autophagy function and is also involved in the inhibition of endothelial cells autophagy under laminar shear stress conditions (Nagy *et al.*, 2006; Fernandez *et al.*, 2009; Caza *et al.*, 2014). The loss of endothelial autophagy promotes reactive oxygen species generation and thereby dysregulation of vascular function (Lavandero *et al.*, 2015). Aligned with these observation, our data indicate that inhibition of Rab4 activity has significantly increased the isoproterenol-stimulated smooth muscle cell migration. However, depletion of Rab4 activity level has an inhibitory effect on VSMC proliferation. VSMCs subjected to AngII treatment showed a significant increase in both proliferation rate and peak-phosho ERK1/2 levels upon Rab4 overexpression. Isoproterenol-stimulated ERK1/2 activation was increased upon Rab4 inhibition in VSMCs (Fig. 4.7). The activation of ERK1/2 was unaffected by β_2AR biased agonist carvedilol. In contrast, Rab4 overexpression augmented ERK1/2 phosphorylation in response to stimulation with β_2AR biased agonist suggesting that Rab4 may selectively regulate the β-arrestin dependent signaling. However, other components of signaling pathway that localize to the endocytic pathway may be also disrupted by Rab4 inhibition, such as Akt or PDK1. These findings suggested key differences in the mechanism of Rab4

Figure 4.7: Schematic diagram of the proposed role of Rab4 in the mechanism of hypertension

Rab4 is a rate-limiting factor in the regulation of expression and function of both β_2A and AT_1 receptors. Primary VSMCs subjected to Rab4 inhibition show activation in ERK1/2 signaling and migration responses to $G\alpha_s$-mediated receptor stimulation. On the other hand, Rab4 overexpression leads to an increase in Ca^{2+} release from intracellular store and alteration in ERK1/2 signaling and proliferation and migration responses to $G\alpha_{q/11}$-coupled GPCR activation.


VASOCONSTRICTION
VASODILATATION
VSMC
Gαq
Gαq
Gαs
Gαs
Rab4
Rab4
PI-PLC
AC
Ca2+
Resensitization
cAMP
ERK
ERK
Cell Proliferation
Cell Migration
Cell Migration
HYPERTENSION

GTPase action on β_2A and AT_1 receptor-mediated signaling linked to both short and long-term cellular responses. These two opposing but highly interlinked signaling pathway likely affect each other and this has direct consequences on vascular smooth muscle cell function.

However, the effects of Rab4GTPase on the GPCR recycling and signaling on the vascular function have not been studied mainly because suitable *in vivo* experimental models do not exist yet. Herein, we have developed a vascular specific dominant inhibitory Rab2S22N transgenic mice to enable definition of the functional role of Rab4 in the hypertension. We observed an age-dependent onset of hypertension in Rab4S22N mice that is maintained throughout the lifespan of the animals. The development of an *in vivo* model of vascular-targeted Rab4 inhibition and the documentation of the hypertensive phenotype in these transgenic mice is the first evidence for the existence of a causal relationship between alteration in Rab4 activity and vascular GPCR signaling. Further characterization of this mouse model is needed in order to establish the molecular mechanisms responsible for alterations in vascular GPCR signaling. Overall, our *in vitro* and *in vivo* models indicate that the loss of Rab4 function is positively correlated with β_2-adrenergic stimulated adenylyl cyclase activity and inversely correlated with blood pressure elevation.

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CHAPTER 5:

DISCUSSION

5.1 Summary

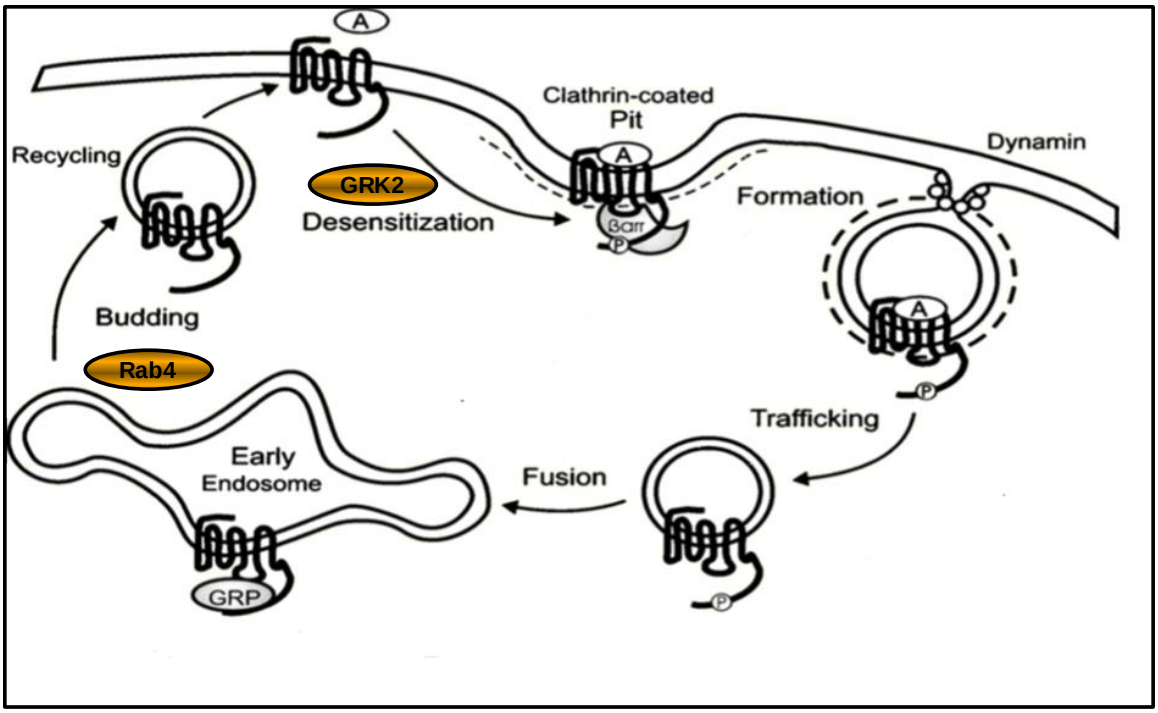
An important component in the regulation of GPCR signaling cycle is represented by the agonist-induced receptor desensitization and internalization from the plasma membrane to the internal endosomal compartments of the cell where GPCRs are dephosphorylated and recycled back to the plasma membrane as functional receptors. However, these processes do not occur in isolation, but rather simultaneously with each cycle of ligand-receptor interaction (Fig 5.1). Since GPCRs play an essential role in the regulation of cardiovascular function, a better understanding of their activity and intracellular trafficking through endosomal compartments can provide insights on the complex mechanism of cardiovascular disease.

Among the various cellular defects associated with the inception of hypertension, alterations in expression and function of GRK2 play an important role. In this thesis, we have attempted to determine whether the genetic knockdown of GRK2 expression leads to a selective sensitization of GPCR-$G\alpha_{q/11}$ protein-mediated signal transduction that is potentially responsible for the hypertensive phenotype. Furthermore, our goal was to elucidate whether Rab4-dependent regulation of GPCRs trafficking plays a significant role in the development of the hypertensive state.

Chapter 2 has clarified the link between changes in GRK2 expression and altered vascular reactivity. Since the targeted disruption of the GRK2 gene in mice is known to result in an embryonic lethal phenotype (Jaber *et al.*, 1996), we had universally knockdown but not knockout GRK2 expression *in vivo*. The shGRK2 mice displayed intrauterine growth-deficient phenotype with lower body mass indices. Our time course observations documented an age-dependent onset of hypertension. The pre-hypertensive phase begins

Figure 5.1: Schematic diagram of the GPCR recycling-cycle

Agonist (A) induced GPCR-desensitization starts off with receptor phosphorylation by the GRK2 and the newly added large phosphates will interfere with G protein-coupling and will facilitate the recruitment of adaptor proteins with role in internalization. Once internalized, GPCRs can traffic from clathrin-coated vesicles to early endosomes from where Rab4 promotes their reinsertion into the plasma membrane through a process called resensitization.


A
Clathrin-coated
Pit
Dynamin
Recycling
GRK2
Desensitization
Formation
Barr
P
Budding
Rab4
A
P
Trafficking
Early
Endosome
Fusion
GRP
P

with a significant increase in systolic blood pressure for 8 week-old shGRK2 mice with a full onset of hypertension observed at 3 months of age. The elevated blood pressure was maintained throughout the lifespan of the animals and it was not confounded by stress or anxiety.

Our findings indicate that the extensive loss of GRK2 expression favors an increase in vasoconstriction associated with an increase in peripheral resistance likely due to reduced $G\alpha_{q/11}$-coupled receptor desensitization. Whereas vasodilatation in response to $G\alpha_S$-coupled receptor stimulation was also enhanced in the shGRK2 mice, the hypertensive phenotype was the consequence of the extent of reduction of the $G\alpha_{q/11}$-selective GPCRs uncoupling.

In terms of downstream signaling pathway, we have found that GRK2 inhibition modulates the AKT and ERK1/2 pathways and these responses are altered with age and progress with the generation of hypertensive phenotype as the pre-hypertensive mice do not exhibit a generalized alteration in response to both $G\alpha_{q/11}$- dependent and –independent β-arrestin-mediated signaling. Further, our data indicate that $G\alpha_{q/11}$- but not $G\alpha_S$-coupled receptor activation leads to increased proliferative and migratory responses of VSMCs and thereby it could be responsible for the increase in peripheral resistance associated with the hypertensive phenotype. Taken together, these data indicate that the balance between the mechanisms regulating vascular tone is shifted to favor vasoconstriction in the absence of GRK2 expression and this leads to the development of hypertension.

In Chapter 3, we have learned that GRK2 inhibition not only contributes to blood pressure elevation – through the activation of vascular AT_1 receptors with consequent vasoconstriction in early stages – but also that the activated intra-renal AT_1 receptors are

capable to maintain the high blood pressure by altering the renal function and further stimulation of the pro-hypertensive mechanisms. Our data indicate that hypertension was initiated through the reduced desensitization of both systemic and intra-renal $G\alpha_q$-mediated vasoconstriction mechanisms as a consequence of decreased GRK2 expression.

Intra-renal injury develops over time with local activation of the RAS components, with a reduction in glomerular filtration rate and dysregulation of renal sodium excretion. Indeed, as blood pressure increases, the expression of renal RAS components increases correspondingly in shGRK2 mice. The chronic activation of RAS not only leads to defective renal-sodium excretion but also potentiates the functional damage of kidney as supported by the presence of proteinuria and low serum albumin levels. In addition, the increased collagen deposition observed especially around renal arteries along with significant reduction in glomerular number reflect the pathophysiological injuries of kidney noticed in our shGRK2 mice.

The treatment with ARB-losartan markedly improved the glomerular filtration rate, supporting the involvement of Ang II-AT_1R axis in the progression of renal injury. Moreover, chronic inhibition of GRK2 expression was associated with differential increases of GRK subtypes, increases that synchronized with the onset of hypertension. In this regard, while GRK5 activation engenders a more compensatory role in attempt to counterbalancing the reduced GRK2-mediated receptor desensitization, GRK4 activation seems to potentiate and further sustain the alteration of kidney excretory function for sodium. Overall, our findings presented in Chapter 3 open up avenues for previously undocumented roles for GRK2 in the pathophysiology of hypertension.

In Chapter 4, we have investigated whether Rab4 and has a distinct role in the regulation of vascular GPCR signaling and thereby on blood pressure homeostasis. We used mutation and transgenic expression of a dominant negative Rab4S22N to define effects of Rab4 on vascular function. The SM22α-Rab4S22N transgenic mice display an age-dependent hypertension phenotype. Hypertension was established in 3-month old mice with significant increases in both systolic and diastolic blood pressures. Moreover, these mice exhibited increased sensitivity to stress-anxiety, implying the activation of the sympathetic responses.

Our data indicate that Rab4 is a rate-limiting factor in the regulation of expression and function of β_2A receptors. Since we showed that the expression of a dominant inhibitory Rab4N121I increased gene transcription activity of β_2A receptors, it can be inferred that Rab4-mediated recycling of the internalized β_2AR plays somewhat of a "housekeeper" with role in the preservation of the number of functional receptors. Alteration of Rab4 activity appeared to lead to decreased of $G\alpha_s$-mediated receptor signaling. Primary VSMCs subjected to Rab4N121expression show alterations in ERK1/2 signaling, which were linked to altered cellular proliferation and migration responses to $G\alpha_s$-coupled GPCR activation. In brief, the developed *in vitro* and *in vivo* vascular specific dominant inhibitory Rab4 models represents one of the first and comprehensive attempt to establish causal relationships between the alteration of Rab4 activity and vascular GPCR signaling.

5.2 Physiological Relevance of GPCR Signal Transduction in Blood Pressure: Consequences of Altered GRK2 Expression on Hypertension

Since GRK2 gene ablation translates into fatality of the mouse embryos beyond E15.5 day (Jaber *et al.*, 1996), it can be inferred that GRK2 plays a cardinal role during the embryonic development. This kinase is expressed in multipotent and migratory cell populations during the early stages of embryogenesis and since after E15.5 day GRK2 was found in heart, liver, lung primordia and motor neurons, its significant contribution to the embryogenic process becomes more than apparent (Sefton *et al.*, 2000). Furthermore, GRK2 is a positive regulator of Hedgehog (Hh) signaling with important effects on the embryonic development as well as multiple pathophysiological processes (Zhao *et al.*, 2016).

In our transgenic mouse model, the targeted knockdown of GRK2 gene expression has translated into a growth-deficient phenotype with lower body mass indices and a significant decrease of adipose mass. The shGRK2 mice show indications of intrauterine growth retardation since the positive transgene pups at E17.5 were significantly smaller in size and had lower body with a significant post-natal loss. Otherwise, our shGRK2 transgenic mice developed normally with the exception of a significant decreased in kidney weight-to-body weight ratio. However, the reason for which shGRK2 mice had a kidney phenotype with no other organ abnormalities remains to be determined. Emerging evidences suggest the existence of a direct correlation between kidney size-nephron number and disease susceptibility in humans (Nyengaard and Bendtsen, 1992; Keller *et al.*, 2003; Hoy *et al.*, 2006; Matsuoka *et al.*, 2006).

According to the Barker-Brenner hypothesis (Brenner *et al.*, 1988; Barker *et al.*, 1989), the onset of hypertension could be a consequence of certain in-utero events. This theory is supported by the fact that infants with low birth weight are more prone to develop hypertension and metabolic pathologies (obesity, diabetes) at adulthood (Barker *et al.*, 1989; Eriksson *et al.*, 2000). Animal models have also shown that maternal malnutrition leading to small birth weights can be linked to elevated blood pressure and salt sensitivity develop during adult life (Woods *et al.*, 2004). Based on these findings, Brenner and his colleagues (Brenner *et al.*, 1988) have proposed that hypertension is the result of congenitally low nephron numbers that are common in newborns with low weight as well as those characterized by intrauterine retardation (Zandi-Nejad *et al.*, 2006). This correlation between low nephron numbers and hypertension has been later reinforced by studies performed on both animals (Woods *et al.*, 2004) and human subjects (Keller *et al.*, 2003; Hughson *et al.*, 2006).

However, while the existence of the correlation was verified, its underlying mechanisms remain more or less obscure. One of the known implications of low nephron count in offspring of malnourished mothers are the pre-glomerular arteriolar disease and tubule-interstitial inflammation (Tapia *et al.*, 2003; Woods *et al.*, 2004). The experimental models involving spontaneously hypertensive rats showed that in addition to the low nephron count and the consequential elevated blood pressure (Woods *et al.*, 2004), animals were also characterized by reductions in afferent arteriolar lumen leading to renal hypoxia and interstitial inflammation (Welch *et al.*, 2001; Rodriguez-Iturbe *et al.*, 2002). These suggest that hypertension might be an outcome of a preexisting renal microvascular injury and inflammation. Along this line of thoughts, low nephron count becomes a

catalyst/accelerator, rather than the root cause of hypertension that might be a consequence of the renal microvascular disease.

In terms of ethnical background, it was shown that low nephron count correlates well with essential hypertension in young Caucasians (Keller *et al.*, 2003). Since similar observations can be extended to White Europeans, White Americans (but not African Americans) and Australian Aborigines (Douglas-Denton *et al.*, 2006; Hughson *et al.*, 2006), it might be inferred that while low nephron number might constitute a strong risk factor for hypertension in young Caucasians, its effect might be superseded by other mechanisms in other populations (*e.g.*, African American).

GRK2 modulates multiple cellular responses through GPCR desensitization and alterations in GRK2 expression have been associated with hypertension. Consequently, alterations in GRK2 expression are proposed to induce a shift in the balance between vasoconstrictor and vasodilator mechanisms suggesting that GRK2 may function as a potential molecular mediator contributing to the induction of hypertension. Previous studies indicate that GRK2 protein expression is elevated in lymphocytes from hypertensive patients and is correlated with reductions in the β_2A-receptor stimulated vasodilation (Gros *et al.*, 1997; Gros *et al.*, 2000). In addition, vascular specific overexpression of GRK2 results in mice that display moderate increases in blood pressure (20%) due to impaired β_2AR-mediated vasodilation (Eckhart *et al.*, 2002). Surprinsingly, when these mice were chalanged with a vasoconstrictor agonist (AngII), GRK2-overexpression reduced blood pressure elevation. On the other hand, our data indicate that global knockdown of GRK2 expression results in a mouse that spontaneously develops hypertension (40%), due to alterations in the balance between mechanisms regulating

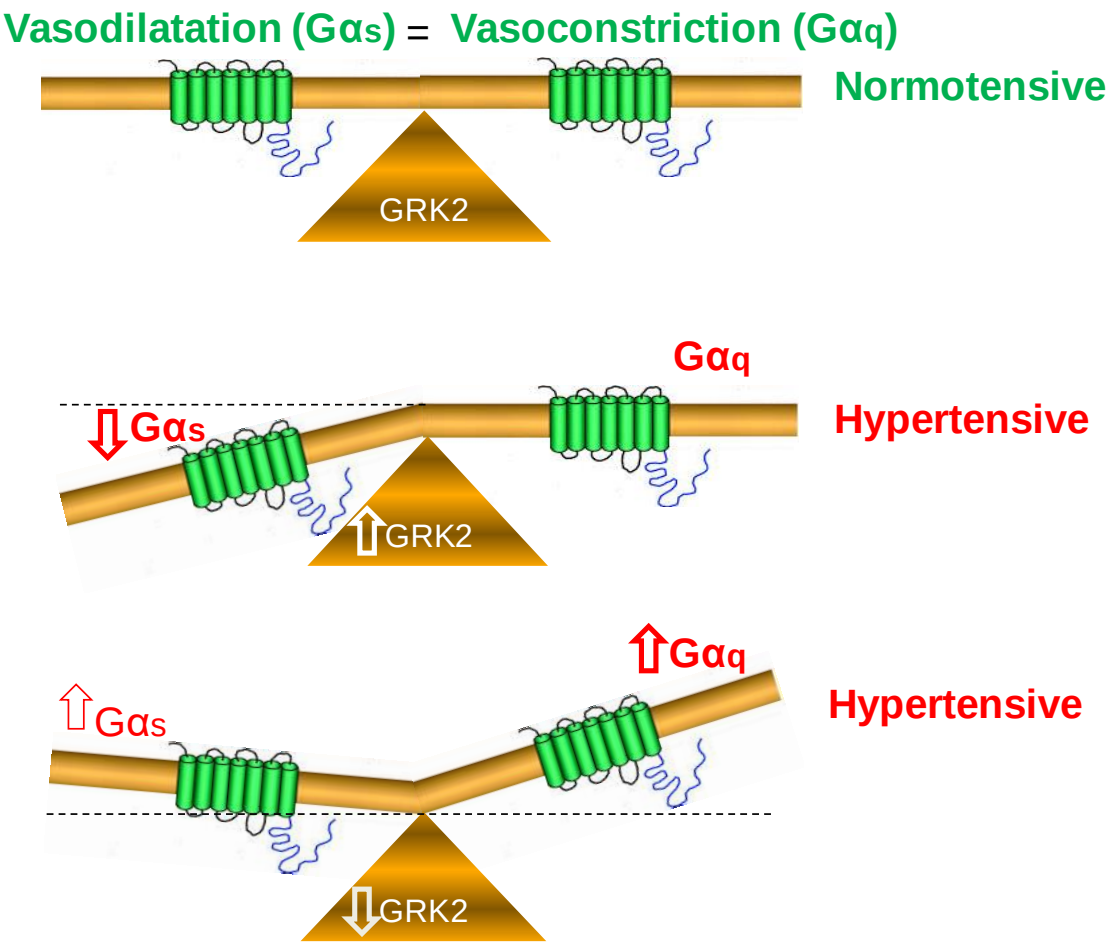
vasodilatation and vasoconstriction. We observed that vasodilatation is enhanced in the shGRK2 mice but – consistent with the hypertensive phenotype – vasoconstriction was also increased and tends to be physiologically predominant (Fig 5.2).

To explain these apparently counterintuitive evidences, we would need to account the following observations: the animal models of altered GRK2 expression are characterized by a gene-targeted mechanism that is not necessarily a "mirror copy" of the pathological GRK2 accumulation. The increased GRK2 protein expression in human hypertension reflects a post-translational mechanism. By contrast, the mechanism of GRK2-targeted alteration of gene expression has more to do with alterations in GRK2 stability such as ubiquitination and phosphorylation (Gros *et al.*, 1999; Penela *et al.*, 2001). On the other hand, GRK2 is a multivalent and multipotent kinase with multiple regulatory roles which has been demonstrated to interact and regulate several substrates through its kinase activity, RGS and PH domains. GRK2 mediates the RGS homology domain phosphorylation-independent desensitization of $G\alpha_{q/11}$-coupled GPCRs only. As such, GRK2 may have the unique capacity to block $G\alpha_{q/11}$-coupled GPCR signaling via its interaction with $G\alpha_{q/11}$.

Thus, the behavior of the analyzed shGRK2 hypertensive phenotype could be the result of GRK2 suppression that might – in turn – reduce the $G\alpha_{q/11}$-selective uncoupling of $G\alpha_{q/11}$-coupled GPCRs by the GRK2 RGS homology domain, a pattern that is not observed for $G\alpha_s$-coupled GPCRs (Ferguson, 2007). $G\alpha_s$-coupled GPCRs might be more responsive to small increases in GRK2 expression due to the loss of phosphorylation-dependent desensitization whereas reductions in GRK2 expression will upregulate both $G\alpha_s$- and $G\alpha_{q/11}$-GPCR signaling.

Figure 5.2: Schematic diagram of the role of GRK2 in the mechanism of hypertension

Alterations in GRK2 expression are proposed to induce a shift in the balance between vasoconstrictor and vasodilator mechanisms regulating vascular tone and this leads to the development of hypertension. GRK2 overexpression shifts the balance towards decreased vasodilatation with intact vasoconstrictor responses. In the absence of GRK2, the balance is shifted to favor vasoconstriction, but with enhanced vasodilatation responses.


Vasodilatation (Gαs) = Vasoconstriction (Gαq)
Normotensive
GRK2
Gαq
Gαs
GRK2
Hypertensive
Gαq
Gαs
GRK2
Hypertensive

Our observations suggest that the loss of GRK2 expression favor an increase in vasoconstriction likely due to reduced $G\alpha_q$-mediated receptor desensitization since $G\alpha_S$-coupled GPCRs that control vasodilatation are regulated slowly by GRK2-dependent phosphorylation whereas $G\alpha_q$-coupled GPCRs are regulated by both GRK2 phosphorylation-dependent and -independent mechanisms. Along these lines, it appears that since both increases and reductions in GRK2 levels are not desirable – due to their negative influence on cell functions – newer therapies will need to attempt to restore GRK2 functionality by accounting for its widely dissimilar roles in various cellular compartments. Regardless of the avenues to be pursued in the future, it is reasonable to expect that therapies to target GRK2 activity, rather than receptor inhibition, will be more effective in the treatment of hypertension.

Since $G\alpha_q$ represents a common driving force behind both cardiac hypertrophy and hypertension, it is possible that the selective inhibition of $G\alpha_q$ will provide new avenues in the treatment of cardiovascular disease. It is important to note that the activated $G\alpha_q$ will interact with the N terminus of GRK2 that – under *in vitro* conditions – is capable to inhibit the $G\alpha_q$-mediated activation of PLCβ (Carman *et al.*, 1999). The characteristics of this inhibition (enhanced by the presence of the agonist, occurs not only after GPCR stimulation but also in the presence of constitutively activated $G\alpha_q$) suggests that it might be a consequence of the direct interaction with $G\alpha_q$ (Carman *et al.*, 1999). In turn, this interaction might be caused by a crucial proline residue that is present in $G\alpha_q$, but not in $G\alpha_i$ or $G\alpha_S$ (Day *et al.*, 2004; Tesmer *et al.*, 2005). Because of this particularity, GRK2 is prone to bind with the effector binding domain of $G\alpha_q$ that is responsible for $G\alpha_q$ associations with PLC proteins as a prerequisite for downstream signaling (Tesmer *et al.*,

2005; Lyon *et al.*, 2014). Therefore, it is possible that a more in-depth investigation of distinct protein-interacting domains of GRK2 will suggest new GRK2-based therapies capable to prevent hypertension and heart failure and thereby will highlight the functional relevance of GRK2 interactions.

The complexity of cellular responses elicited upon activation of GPCRs are tightly regulated by phosphorylation status of the receptors mediated by GRKs. According to the GRK subtypes, phosphorylation can be regulated differentially leading to distinct phosphorylation profiles or "signalling barcodes" (Butcher *et al.*, 2011). In this way, the patterning of receptor phosphorylation determines the conformation of the bound β-arrestins and, subsequently, its functional capabilities. Therefore, by adopting a specific "phosphorylation profile," a receptor could encode a particular signaling pathway (Kim *et al.*, 2005; Ren *et al.*, 2005; Zidar *et al.*, 2009). Members of the GRK family can phosphorylate different sites on the same receptor whereas some receptors are actually regulated by a single GRK (Torrecilla *et al.*, 2007; Tobin *et al.*, 2008). In our model, chronic inhibition of GRK2 expression was associated with differential increases in the other GRK subtypes (GRK3, GRK4, GRK5, and GRK6), increases that were synchronized with the onset of the hypertensive state. Thereby, the follow-up question is whether the activation of these kinases has a distinct role on the mechanisms of hypertension or their increase induces a compensatory role in the attempt to balance the attenuation in GRK2-mediated receptor desensitisation. Interestingly, while increased GRK5 levels positively correlates with increased blood pressure, GRK3 and GRK4 seems to further sustain and potentiate the GRK2-dependent mechanism of hypertension. As such, changes in GRKs levels may reduce or enhance GPCR phosphorylation and thereby subsequently altering

the downstream signaling pathway. It is worth to emphasise that even though GRKs isoforms are expressed at low levels in basal conditions, their expression might be important and relevant under pathological conditions. The challenge will remain to fully comprehend the role played by GRKs in the disease state.

5.3 Future Directions

We have learned that shGRK2 transgenic mice are characterized by an intrauterine growth restriction with a significant post-natal loss. Therefore, future studies could seek to determine if GRK2 mice can be used as a model of intrauterine growth restriction (IUGR) phenotype for studying of the mechanisms linking birth weight and hypertension.

From a strict diagnosis standpoint, IUGR is usually defined through a fetal weight that falls under the 10th percentile of the normal body weight. However, this rather simple definition tends to include – in addition to the targeted pathologies – underweight individuals whose occurrence is normal in a population. IUGR is a complex phenomenon caused by the intricated effects of multiple maternal, fetal, placental and environmental factors. While the intrinsic correlation between the low oxygen/nutrients supply and IUGR is widely acknowledged, the mechanistic substrate of this link is still largely unknown. Since in addition to nutritive, placental and hormonal factors, IUGR dysregulations are often associated with gene mutations and expressions (Mukhopadhyay *et al.*, 2010; Chen *et al.*, 2011; Mando *et al.*, 2011; Chelbi *et al.*, 2012; Borzsonyi *et al.*, 2013), it can be inferred that the investigation of the related pathways by means of engineered transgenic models might shed some light on the underlying molecular mechanisms.

Importantly, the use of a mouse model of IUGR will allow us to address novel hypotheses related to the developmental origins of hypertension. Since the relationship

between GRK2 levels and impaired vasoconstriction responses is causal for hypertension, it would be interesting to decipher if this trait is also present in the fetal placental vasculature. At early development stages, GRK2 is express in cells that are multipotent, undifferentiated that undergo shape changes and are associated to migratory processes (Sefton *et al.*, 2000). At later development stages, GRK2 is present in several organs and tissues supporting is broader role in embryogenesis. GRK2 is unique among the GRK family members in that no embryos survived past embryonic day E15.5 under GRK2 knockout phenotype (Jaber *et al.*, 1996; Matkovich *et al.*, 2006). A greater understanding of the essential function of GRK2 in the developing embryo would be of significant interest especially since limited data are available. Therefore, future studies could seek to determine what is the exact relationship between GRK2 downregulation and renal development. Further, since our preliminary data indicate a loss in the number of nephrons during embryonic development, it would be important to determine the conditional genetic modifications involved in the cessation of nephrogenesis in shGRK2 mice. The investigation of the complex role of GRK2 on embryogenesis could grant us a better understanding of the role played by GRK2 under pathological conditions.

We have observed that down-regulation of GRK2 expression significantly decreases age-related adiposity in shGRK2 mice compared to wild type littermates. Moreover, the shGRK2 mice display higher respiratory exchange ratio and significant increases in both total and ambulatory activity measurements. One possible future study could attempt to investigate the molecular mechanisms responsible for these outcomes and it would be interesting to evaluate whether the lean phenotype observed in the shGRK2 mice is caused by a direct effect of GRK2 on energy homeostasis. Our data also indicate that several genes

with role in lipid catabolism are upregulated in shGRK2 mice. In support of this idea, there is evidence that $GRK2^{+/-}$ mice display an increase in gene expression related to thermogenesis as well as enhanced lipolysis rate. Moreover, the overexpression of GRK2 can suppress lipolysis in white adipose tissue and energy burning in brown adipose tissue (Vila-Bedmar *et al.*, 2012). As such, future studies could analyze the specific role of GRK2 on cellular metabolism with potentially relevant targets in the treatment of obesity and associated metabolic disorders.

The availability of animals models makes possible the study of molecular mechanisms that influence the function of genes implicated in disease and disorders. The development of a transgenic mouse model of specific vascular-targeted Rab4 inhibition presented in Chapter 4 and the documentation of the hypertensive phenotype highlights a number of interesting questions that could be pursued in the future. A greater characterization of this model would enable us to understand the pathophysiological role of Rab4 modulated GPCR internalization/recycling. Therefore, it would be of significant interest to assess vascular reactivity in response to vasodilator and vasoconstrictor agonists. We could further investigate the $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ signaling via G protein and β-arrestin-mediated pathways in primary cultures of VSMCs derived from Rab4S22N transgenic mice. Since differences in Rab4 protein expression levels can vary from on cell type to the other might be interesting to assess intracellular trafficking and signaling patterns of GPCRs in primary cultures of endothelial cells obtained from the Rab4S22N transgenic mice. To enable definition of the functional role of Rab4 on long term cellular responses, 3D scaffold methods can be employed to assess cellular proliferation, differentiation, migration, and adhesion responses.

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