ACTIVATION OF NOVEL SIGNAL TRANSDUCTION PATHWAYS BY FP RECEPTORS: THE G-PROTEIN COUPLED RECEPTORS FOR PROSTAGLANDIN F_{2A}

by

Wei Xu

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2007

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Wei Xu

Entitled: Activation of Novel Signal Transduction Pathways by FP Receptors: the G-Protein Coupled Receptors for Prostaglandin $F_{2\alpha}$ and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

John W. Regan	Apr. 12th, 2007 Date
John W. Bloom	Apr. 12th, 2007 Date
Tim Bowden	Apr. 12th, 2007 Date
Roger Miesfeld	Apr. 12th, 2007 Date
Dan Stamer	Apr. 12th, 2007 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College. I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

John W. Regan	Apr. 12th, 2007
Dissertation Director	Date

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SIGNED: Wei Xu

ACKNOWLEDGEMENTS

First, I would like to thank Dr. John W. Regan for the support and guidance he has provided throughout my doctoral studies. Dr. Regan has given me many opportunities to expand my horizons by allowing me to have a lot of freedom in my research. I venerate Dr. Regan's intelligence, work ethic, scientific integrity as well as attention to detail; they are second to none and it is easy to see why he is very successful.

I would also like to thank my committee members Doctors John Bloom, Tim Bowden, Roger Miesfeld and Dan Stamer for their scientific and professional support.

I would like to thank Dr. Chiling Chou for her time, experience and prospect in helping me with my studies. We worked together to get the results presented in this thesis. Without her help, I can not image how I can achieve so much in my graduate study.

I would like to thank all the other folks in the Regan lab; Alfred, George, Dinesh, Hiromichi, Tony, Chi-ling and Amy for their help over the years. I would also like to thank Dr. Vallancourt for his help in my projects. It is his idea and vision to help this project take off.

Last, and certainly not least, I would like to thank my parents, Kanglin Xu and Yixin Shen as well as my wife Zhou and son William for their support over the years.

DEDICATION

This dissertation is dedicated to my family, especially my parents and parents-in-laws, who over the years have supported my efforts with great patience, support and love. I would also like to thank my wife Zhou and son William for their support over these years.

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LIST OF ABBREVIATIONS

COX-1 = Cyclooxygenase 1

COX-2 = Cyclooxygenase 2

GPCR = G-protein coupled receptor

NSAID = Non-steroidal anti-inflammatory

PCR = Polymerase chain reaction

PGD_2 = Prostaglandin D_2

PGE_2 = Prostaglandin E_2

$PGF_{2\alpha}$ = Prostaglandin $F_{2\alpha}$

PGG_2 = Prostaglandin G_2

PGH_2 = Prostaglandin H_2

PGI_2 = Prostaglandin I_2

PKC = Protein kinase C

PLA_2 = Phospholipase A_2

TM = Transmembrane

TXA_2 = Thromboxane A_2

RT-PCR = Reverse transcriptase polymerase chain reaction

GAPDH = Glyceraldehyde-3-phosphate

TCF= T cell factor

HIF-1α= hypoxia inducible factor 1α

Cyr61= cysteine-rich, angiogenic inducer 61

CTGF= connective tissue growth factor

EGR-1= early growth response factor 1

DN= Dominant negative

ABSTRACT

Prostaglandin F2 alpha ($PGF_{2\alpha}$) is an arachidonic acid metabolite which plays an important role in cardiac hypertrophy and cancer. $PGF_{2\alpha}$ is known to activate intracellular signaling pathways by interactions with its cognate G-protein coupled receptor named the FP prostanoid receptor. To date, the signal transduction pathways by which the FP receptor regulates gene expression have yet to be fully characterized. In this dissertation, multiple novel signal transduction pathways by the activation of FP prostanoid receptors involved in the regulation of gene expression have been identified and characterized.

To study FP-dependent gene regulation, cDNA microarray technology was applied using HEK293 cells expressing FP receptors as a model. More than 150 genes, which could be classified into diverse functional groups, were identified to be significantly regulated through the stimulation of FP receptor in the cDNA microarray analysis. To confirm the results from microarray analysis, 20 significantly regulated genes from cDNA microarray analysis were subjected to Northern blot analysis. The expression profile of 14 out of these 20 genes was in agreement with that of cDNA microarray data.

One of the 14 genes is early growth response gene 1 (EGR-1). EGR-1 is a transcription factor which has been shown to play important roles in the cardiac hypertrophy. Another gene identified is connective tissue growth factor (CTGF). CTGF belongs to CCN family, which play an important role in cancer angiogenesis. Cysteine-rich, angiogenic inducer-61 (Cyr61) was another member of CCN family. Gene regulation of Cyr61 through the

FP receptor has previously been reported. FP receptor mediated gene regulation of the above three proteins was confirmed using Western blotting analysis.

Following confirmation of FP receptor mediated gene regulation of EGR-1, Cyr61 and CTGF, the pathways responsible were dissected. We found that FP can activate Ras, which in turn activates C-Raf. Activation of C-Raf activates MEK1/2, and leads to the up-regulation of EGR-1. On the other hand, the studies demonstrated that activation of Ras through the FP receptor activate B-Raf, which can lead to the TCF/β-catenin pathway. TCF/β-catenin pathway regulates the expression of Cyr61. We also found that HIF-1α induced by the activation of FP receptor is involved in the regulation of CTGF expression. Moreover, we found that reactive oxygen species (ROS), a well-known activator of HIF-1α, is involved in the activation of TCF/β-catenin pathway, which leads to the gene regulation of HIF-1α.

In conclusion, these studies have identified previously unknown signaling pathways and novel downstream effectors that are regulated by the FP prostanoid receptors. The identification of these novel interactions between the pathways activated by the FP receptor may have future applications in the treatment of heart disease and cancer.

CHAPTER ONE

INTRODUCTION, PURPOSE AND AIMS

1.1 Introduction

Prostaglandin is an important drug target. For example, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDS), which are used every day in modern life, lies in their ability to inhibit prostaglandin synthesis to control the pain (Vane et al., 1971). Prostaglandins belong to the group of chemicals named autacoids, which are thought to be extremely susceptible to metabolic degradation, thereby limiting their influence on the cells producing them and the cells surrounding the cells producing them. Autacoids can be divided into two major divisions, eicosanoids and the modified phospholipids.

Eicosanoids are oxygenated products of polyunsaturated long chain fatty acids such as arachidonic acid. Eicosanoids can be classified as prostaglandins, leukotrienes, epoxides, and isoprostanes, depending on the enzyme systems used to metabolize arachidonic acid to generate the end products. Thus, the leukotrienes are synthesized by the action of lipoxygenases, epoxides by the action of epoxygenases, isoprostanes by the action of free radicals and prostaglandins by the action of prostaglandin H synthase.

Prostaglandins were discovered in the 1930s as a chemical mainly present in seminal fluid and accessory reproductive glands that caused strips of human uterus to relax and contract upon contact. Euler coined the name prostaglandin in 1935 to denote lipid soluble acids produced by the prostate glands. Various kinds of prostaglandins were soon found, characterized, and chemically synthesized. These prostaglandins included PGD_2,

PGE_2, $PGF_{2\alpha}$, prostacyclins, and thromboxanes. They are biochemically synthesized by the actions of phoshpholipase A_2 on membrane phospholipids. The released arachidonic acid product is then rapidly oxygenated and metabolized by enzyme systems such as the cyclooxygenases, or the lipoxygenases. The action of cyclooxygenases on the arachidonic acid generates PGH_2. PGH_2, which is immediately chemically unstable, serves as a substrate for various synthases, resulting in the synthesis of a multitude of prostaglandins. Figure 1.1 outlines the synthetic pathway of prostaglandins.

Cyclooxygenases are the key enzyme in the synthetic pathway of prostaglandins, which regulates the production of prostaglandins. Cyclooxygenases themselves exist as two isoforms named as COX-1 and COX-2. COX-1 is thought to be a housekeeping enzyme is constantly expressed. COX-2, however, is known to be inducible and is the major contributor precipitating an adverse inflammatory response (Williams et al., 1996). COX is the drug target of NSAIDs such as aspirin. These drugs are conventionally used to treat mild to severe conditions, and offer analgesic, antipyretic, and anti-inflammatory effects. Recently, it has been found that long-term use of low doses of aspirin is also associated with reduced incidence of myocardial infarction and lower incidence of colon cancer.

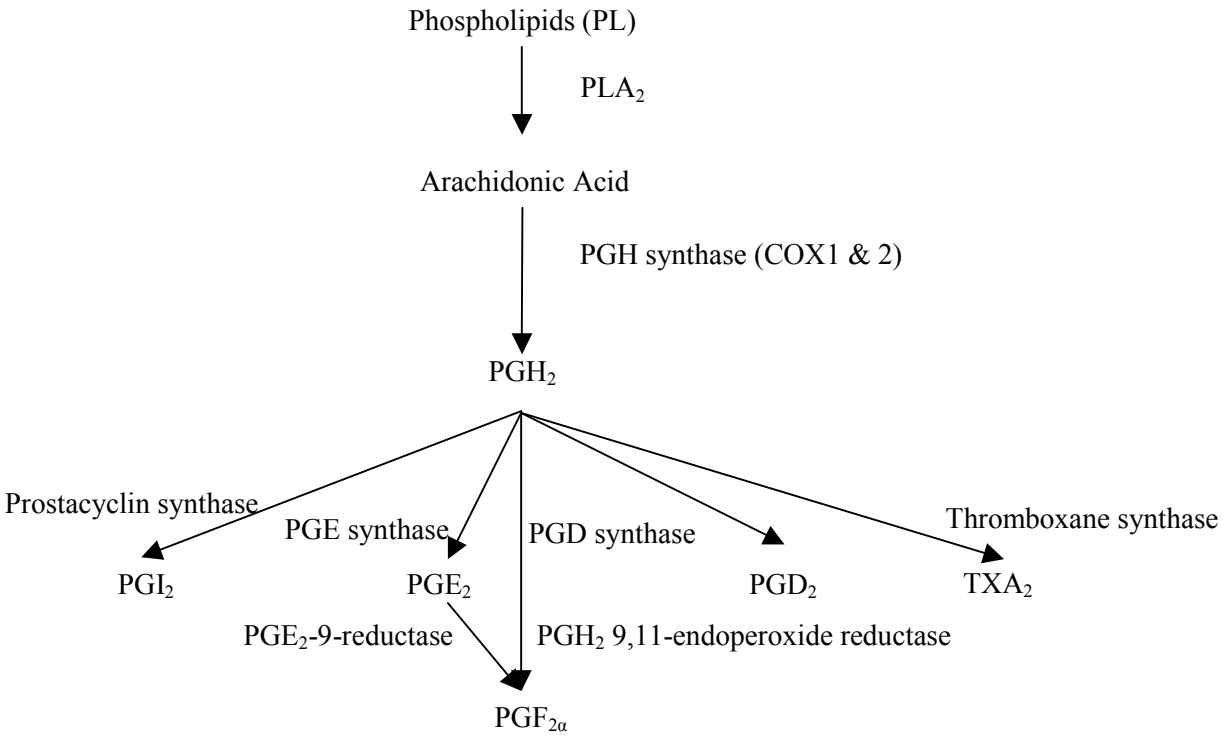

Figure 1.1 Schematic pathways of prostaglandin synthesis from arachidonic acid release and metabolism. Arachidonic acid is produced from phospholipids (PL) by PLA_2. COX then converts the arachidonic acid to prostaglandins. Individual isomerases (e.g. $PGF_{2\alpha}$ synthase) convert unstable PGH_2 to the five major prostanoid subclasses, PGD_2, PGE_2, PGI_2, $PGF_{2\alpha}$ and TXA_2.

Although COX inhibitors are wildly used in clinic, these drugs have some side effects. The most recent example is the withdrawl of COX-2 inhibitor rofecoxib (Vioxx) from the market because of its side effect in heart. So, drug targeting individual prostaglandins, the product of COX, may be necessary to circumvent these side effects. Prostaglandin receptor antagonists may be good candidates. To develop the prostaglandin receptor antagonists, it is necessary to identify the receptors to which prostaglandins bind.

1.2 Prostanoid receptors

It is now known that the prostanoids activate signaling processes by activation of specific G-protein coupled receptors (GPCRs), which are seven transmembrane spanning proteins that have an extracellular amino terminus and an intracellular carboxyl terminal tail. They are coupled intracellularly to heterotrimeric G-proteins. The binding of a ligand to its receptor results in a conformation change (usually in the alpha helices of the transmembrane domains). This change will activate the G-protein heterotrimer (GDP exchanged with GTP), resulting in the transduction of the extracellular stimulus into an intracellular response through the activation of second messengers, such as calcium and protein kinases.

There is at least one GPCR for each of the five main prostaglandins, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A. Thus there are five major subdivisions of the prostanoid receptors that have been defined pharmacologically. They are the DP, EP, FP, IP, and TP receptors which are activated by the PGD2, PGE2, $PGF_{2\alpha}$, PGI and TxA_2 respectively

(Coleman et al., 1994). Functionally, it has been shown that both transmembrane and extracellular regions of the prostanoid receptors are involved in ligand binding. Although each prostanoid has the highest affinity to its cognate receptor, there is some cross-reactivity to other receptors within the family.

The use of recombinant DNA technology has facilitated the cloning of cDNAs encoding all the prostanoid receptors and elucidating signal transduction cascades elicited by the activation of the various receptors (Pierce et al., 1998). Multiple subtypes of EP receptors has been identified that are each encoded by distinct genes. The subtypes of EP receptor isoforms are the EP_1, EP_2, EP_3 and EP_4, and these account for the differential tissue-specific effects elicited by PGE_2 analogs. For the downstream signaling, the DP_2, IP, EP_2 and EP_4 prostanoid receptors are coupled to Gs and result in an activation of adenylate cyclase. The EP_3 receptors are coupled to Gi to produce an inhibition of adenylate cyclase. The EP_1, FP, and TP are coupled to Gq, which stimulates phospholipase C to produce inositol triphosphate and diacylglycerol.

1.3 FP receptors

The FP receptors are GPCRs whose physiological agonist is $PGF_{2\alpha}$. FP receptors were first cloned from a human kidney cDNA library and FP receptor gene encodes a protein of 359 a.a. (Abramovitz et al., 1994). Since then, FP has been cloned from multiple species. For example, ovine FP was cloned in 1995 (Graves et al., 1995). FP was also cloned from mouse and bovine (Sugimoto et al., 1994; Sakamoto et al., 1994). FP

isoforms generated by the different mRNA splicing were also cloned. The first FP isoform to be unveiled was the ovine alternative splice variant (Pierce et al., 1997). This FP isoform was designated FP_B compared to the original isoform, which was further defined as FP_A. The ovine FP splice variants are identical in amino acid sequence until they diverge at a tyrosine residue nine amino acids into the carboxy tail. The FP_A isoform continues for 46 amino acids downstream of the splice site while the FP_B has only one additional residue (isoleucine) resulting in a truncated receptor relative to the FP_A. The second FP receptor isoform was cloned from bovine corpus luteum that is created from alternative mRNA splicing at the 6TM site (Ishii et al., 2001).

FP has been shown to be present in numerous tissues from a variety of different species. Northern blot analysis of 12 human tissues revealed mRNA expression in the heart, skeletal muscle, colon, kidney, small intestine, placenta and lung (unpublished data). FP also has been shown to express in human granulosa cells and osteoblasts, the corpus luteum, myometrium as well as smooth muscle and eye tissues (Anthony et al., 1998; Mukhopadhyay et al., 1999).

Considering the ubiquitous presence of FP in the organs, it is not surprising that FP plays some important roles in many physiological processes. For example, FP has been found to be the key in the uterus contraction during parturition (Sugimoto et al., 1997). FP has also been shown to play key role in corpus luteum regression (Olofsson et al., 1994). FP is not only involved in the normal physiological process, but also plays important roles in

several diseases. One example is that FP can trigger heart hypertrophy (Adams et al., 1996). FP receptor has also been shown to be important in endometrial adenocarcinomas (Sales et al., 2005).

To better understand the biological functions of FP receptors, it is necessary to identify signal transduction pathways initiated from FP receptor. At first, FP receptors were linked to G_q protein and the downstream components of G_q protein kinase C (PKC) and calcium (Abramovitz et al., 1994; Pierce et al., 1997). However, evidence indicated the existence of signal transduction pathways other than G_q. For example, activation of Rho small G proteins, which probably is regulated by $G_{12/13}$ but not G_q, is involved in a cell shape change and actin stress fiber formation induced by the activation of the FP receptor (Pierce et al., 1998). Furthermore, activation of T-cell factor (Tcf)/β-catenin signaling by FP receptors was identified (Fujino et al., 2001) and this activation was shown to be at the downstream of the Rho signal transduction pathway (Fujino et al., 2002). All these signal transduction pathways are summarized in Fig 1.2.

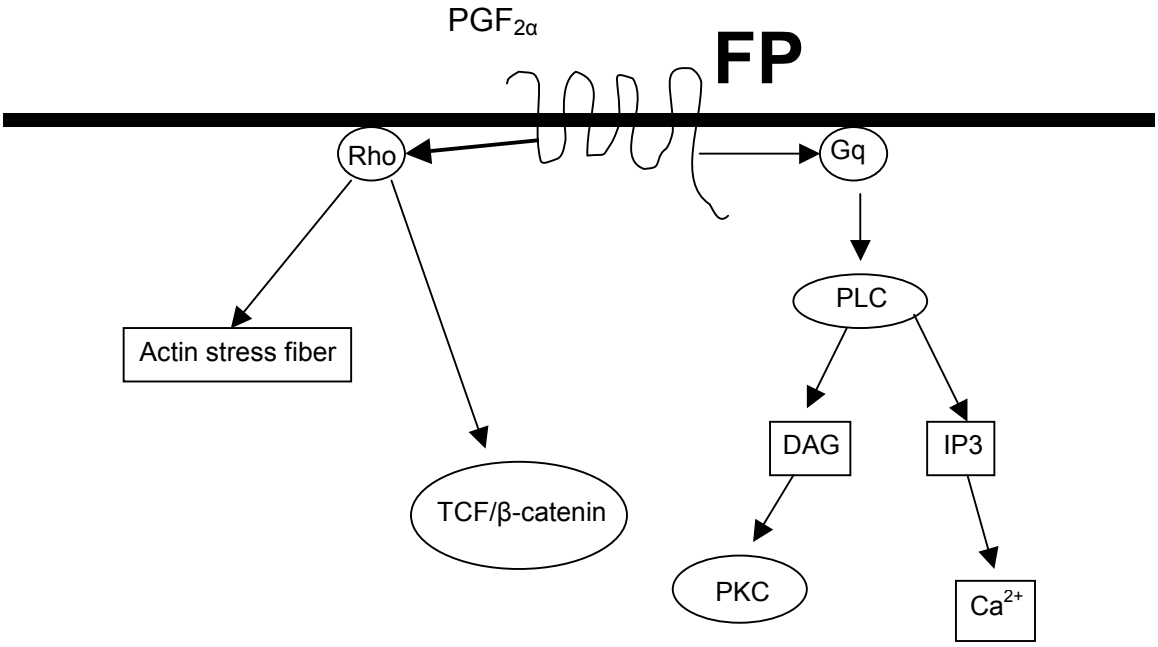

Fig 1.2 The signal transduction pathways through the FP receptor. The FP receptor is capable of coupling to the G_q protein hydrolyzing phosphatidylinositol lipids resulting in activation of PKC and Ca^{2+} mobilization. The FP receptor also activates the small G-protein Rho, resulting in the formation of actin stress fiber. Moreover, the FP receptor can activate the TCF/β-catenin pathway through Rho small G protein.

Gene regulation is an important aspect of signal transduction. Some studies have identified certain genes, which are regulated through the FP receptor, and related signal transduction pathways involved in these regulations. One example is the up-regulation of connective growth factor (CTGF) and cysteine-rich angiogenic protein 61 (Cyr61) by the activation of the FP receptor (Liang et al., 2003). Rho small G protein and MAPK pathways were shown to be involved in these regulations. Another example is the induction of COX-2 through the FP receptor (Fujino et al, 2003). TNF-α was also shown to be induced by activation of the FP receptor (Fujino et al., 2004). But overall, gene regulations mediated by the FP receptor and signal transduction involved in these regulations are not well established.

1.4 Purpose and Aims

The overall purpose of this work was to detail the signal transduction pathways leading to the gene regulation by the activation of FP receptor.

To achieve this goal, four specific aims are set up:

The first aim is to use cDNA microarray technology to identify the genes whose expression were significantly regulated by the activation of the FP receptor in HEK-293 cell lines stably expressing FP receptors (FP cells). To carry out this aim, cDNA

microarray analysis were performed using RNA extracted from FP cells treated with vehicle or $PGF_{2\alpha}$ for different times are extracted.

The second aim is to use Northern blot analysis and Western blot analysis to confirm the gene regulations identified in cDNA microarray analysis in $PGF_{2\alpha}$-stimulated FP cells. Multiple genes, whose expressions were found to be significantly regulated in the cDNA microarray analysis, were chosen and their expression profiles were confirmed at the RNA and protein levels using Northern blotting analysis and Western blotting analysis.

The third aim is to identify the signal transduction pathways leading to the regulation of genes in $PGF_{2\alpha}$-stimulated FP cells. To carry out this aim, several genes, whose regulations in $PGF_{2\alpha}$-stimulated FP cells are confirmed at protein levels using Western blot analysis, were chosen and signal transduction pathways leading to their regulation were dissected using multiple approaches, including specific signal transduction inhibitors and dominant negative constructs targeting specific signal proteins.

The fourth aim is to confirm the signal transduction pathways identified in the FP cells using a system endogenously expressing FP receptor. To carry out this aim, we first identified the cells which endogenously express FP receptor. Then, the genes, whose regulations through the FP receptors have been characterized in FP cells, were chosen to be subject to signal transduction dissection in cells expressing FP receptors endogenously. The methods used for dissection were FP antagonist or specific signal

transduction inhibitors. Cells were pretreated with FP antagonist or specific signal transduction inhibitors followed by treatment with $PGF_{2\alpha}$. We expected that $PGF_{2\alpha}$-stimulated gene regulations in cells expressing FP receptor endogenously are similar to that in FP cells. We also expected FP antagonist and specific signal transduction inhibitors can block these gene regulations, which is similar to that in FP cells.

CHAPTER TWO

IDENTIFICATION OF GENES REGULATED BY THE ACTIVATION OF FP PROSTANOID RECEPTOR UTILIZING CDNA MICROARRAY TECHNOLOGY

2.1 **Introduction**

A cDNA microarray is a collection of microscopic cDNA spots attached to a solid surface, such as glass, plastic or a silicon chip, forming an array for the purpose of expression profiling and monitoring expression levels for thousands of genes simultaneously. This technology is widely used to study the gene regulation at genomic level.

Some studies have been done to elucidate the gene regulation by the activation of FP receptors. One study, for example, focused on the up-regulations of connective growth factor (CTGF) and Cyr61 by the activation of FP receptor (Liang et al., 2003). Another example is the up-regulation of matrix metalloproteinase-1 production by prostaglandin $F_{2\alpha}$ in human gingival fibroblasts (Noguchi et al., 2001). However, a systematic study of gene regulation by the activation of FP receptor has not yet been reported.

To study gene regulation mediated by the FP receptor at genomic level, cDNA microarray technology was applied to study the gene regulation of agonist stimulated HEK293 cells stably expressing the ovine FP_A and FP_B receptor isoforms (FP_A and FP_B cells). We found extensive gene regulation at the level of the whole genome. To confirm the results from microarray analysis, 20 significantly regulated genes from cDNA microarray analysis were subjected to Northern blot analysis. The expression profile of 14 of these 20 genes was in agreement with that of cDNA Microarray data. One of the 14 genes, EGR-1, was subjected to Western blot analysis. The expression profile of EGR-1

at the protein level is the same as that from Northern blot analysis. This is the first report of gene regulation by the activation of the FP receptor at the genomic level.

2.2 Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, bovine serum albumin, hygromycin B, geneticin, gentamicin and Trizol were obtained from Invitrogen (Carlsbad, CA). Anti-EGR-1 antibodies were obtained from Santa Cruz biotechnology (Santa Cruz, CA). Anti-rabbit IgG conjugated with horseradish peroxidase, poly-deoxyinosinic-deoxycytidylic, polyoxethylenesorbitan monolaurate and all other unspecified chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Anti-actin IgG, anti-mouse IgG, Zeta-probe blotting membrane and nitrocellulose membrane were purchased from Bio-Rad (Hercules, CA). $PGF_{2\alpha}$ was obtained from the Cayman Chemical Company (Ann Arbor, MI). [α-^{32}P] dCTP (10 mCi/ml) was from PerkinElmer Life and Analytical Sciences (Boston, MA).

Cell culture. FP_A and FP_B cells were maintained at 37°C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 250 μg/ml geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin.

RNA extraction. Cells were incubated at 37°C with vehicle for 24 hours or 1 μM $PGF_{2\alpha}$ for 15 minutes, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours. After treatment, crude RNA was extracted using Trizol according to the manual. The crude RNA extract was

then purified by Absolute RNA® Miniprep Kit (Stratagene, Cedar Creek, TX). The purified RNA was concentrated by speedvacuum and the concentration was determined by UV spectrum at 260 nm and 280 nm wavelengths.

Microarray analysis. The cDNA microarray fabrication was performed as previously described (Watts et al., 2001). Fluorescent cDNA was made from reverse transcription of 40μg of total RNA in the presence of 50 μM Cy5-dCTP or Cy3-dCTP in a 25 μl volume containing the following: 500 ng oligo(12-18) dT, 1 X Superscript Buffer, 400 U Superscript II, 3.3 U RNAse inhibitor (all from Gibco BRL, Grand Island, NY), 400 μM each of dGTP, dATP, dTTP, 100 μM dCTP, and 10 mM dithiothreitol. The cDNA was purified, lyophylized to dryness, re-suspended in 10 μl hybridization buffer (2 X SSC, 0.1% SDS, 100 ng/μl Cot1 DNA, 100 ng/μl oligo dA), denatured by boiling for 2.5 minutes and hybridized to a microarray for 16 hours at 62˚C for 18 hours. Following hybridization, slides were washed and scanned for Cy3 and Cy5 fluorescence using an Axon GenePix 4000 microarray reader and quantitated using GenePix software. Results were loaded into GeneSpring (Silicon Genetics, Redwood City, CA) and normalized using lowess intensity dependent normalization. The cutoff score for up- or down-regulation was defined as follows: in at least one channel the signal of the genes must be 1.5 times more than the background signal and the signal of genes from $PGF_{2\alpha}$ treated cells should be changed at least 2 times compared with the signal of genes from control cells.

Northern blotting analysis. 10 μg RNA each well was run on 1% denaturing formaldehyde agarose gels, and RNA was transferred to a Zeta-probe blotting membrane after alkali denaturation and neutralization. The membranes were then baked in 80^{o}C for 2 hours and wetted with diethyl pyrocarbonate treated water. Hybridizations were carried out at 42°C for 16 hours with labeled DNA probe in 50% deionized formamide, 10% dextran sulfate, 1% (w/v) SDS, 1 M NaCl and 100 μg/ml denatured salmon sperm DNA. The DNA probe was prepared using Prime-a-Gene® Labeling System (Promega, Madison, WI) followed by purification with PCR purification kit (Gibco BRL, Grand Island, NY). Blots were washed twice at 42°C in 2 x SSC, 0.1% (w/v) SDS for 30 minutes each, and twice at 42°C in 0.1xSSC, 0.1% (w/v) SDS for 30 minutes each.

Immunoblot Analysis. Cells were incubated at 37^{o}C with vehicle for 24 hours or 1 μM $PGF_{2\alpha}$ for 1 hour, 3 hours, 6 hours, 12 hours and 24 hours. Cells were scraped into a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 10mM sodium fluoride, 10 mM disodium pyrophosphate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin, and then transferred to microfuge tubes. The samples were rotated for 30 minutes at 4^{o}C and were centrifuged at 16,000g for 15 minutes. Protein concentrations were determined using a Bio-Rad assay kit (Hercules, CA). For Western blotting analysis using anti-EGR-1 antibody, aliquots of the supernatants containing 20 ~ 100 μg protein were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were

blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.1% polyoxethylenesorbitan monolaurate (TBST) for one hour at room temperature and were then incubated at 4^{o}C for 16 hours with primary antibodies (1:1000 dilution). After the membranes underwent three five-minute washes in TBST, they were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (1:10000 dilution) for 1 hour at room temperature with rotation. The membranes were washed and visualized by enhanced chemiluminescence (Supersignal; Pierce, Rockford, IL). The primary antibodies are anti-EGR-1 (Santa Cruz) and anti-actin antibodies (Biorad).

2.3 Results

Gene expression profile of FP_A or FP_B cells following treatment with $PGF_{2\alpha}$. To characterize gene regulation through the activated FP receptor, cDNA microarray analysis was employed to determine the temporal gene regulation profile of FP_A or FP_B cells following treatment with $PGF_{2\alpha}$. We treated FP_A or FP_B cells with $PGF_{2\alpha}$ for different lengths of time as described in **Experimental Procedures,** then extracted the RNA. The time points were chosen to identify the early, intermediate and long-term responses of cells to $PGF_{2\alpha}$. cDNA microarray hybridization and data analysis were carried out as described in **Experimental Procedures.** The genes shown to be regulated according to the standards in **Experimental Procedures** are summarized in table 2.1. The genes were classified into categories based on functional annotation (as determined by the NCBI Protein Database and SOURCE) and the results are summarized in Fig. 2.1.

This classification revealed that the genes encompassed a wide range of functional groups. The Transcription category (15.5% in Table 2.1) and Signaling category (16.1% in Table 2.1) were the two largest groups. These results suggest that the activation of FP receptors has a broad impact on cell physiology by affecting genes involved in a variety of biochemical pathways and diverse cellular functions.

Northern blot analysis of genes identified to be regulated in cDNA microarray analysis. To validate the microarray data, we used Northern blot analysis to investigate the expression profiles of the genes regulated in cDNA microarray analysis. From Table 2.1, we selected 20 genes whose functions interested us. The Northern blots were performed as described in **Experimental Procedures** and the results are summarized in Table 2.2. In Table 2.2, 14 out of 20 genes showed a similar pattern of gene regulation in both the Northern blots and the microarray analysis. Eight genes, which were found to be regulated in an agonist-dependent manner, were subject to further Northern blot analysis. The data are summarized in Fig. 2.2. To confirm equal loading, the blots were stripped and re-probed with glyceralaldehyde-3-phosphate dehydrogenase (GAPDH). As shown in Fig. 2.2, nearly equal amounts of GAPDH were present in all cell lines and throughout the time course in each Northern blot. The regulation of each gene shown in Fig. 2.2 was agonist-dependent.

Western blot analysis of EGR-1. After Northern blot analysis, we chose EGR-1, one of the genes whose expression profile was confirmed utilizing Northern blotting analysis,

for further confirmation at the protein level. The drug treatment, cell lysate preparation and Western blot analysis were carried out as described in **Experimental Procedures**. The results are presented in Fig. 2.3. To confirm equal loading, the blots were stripped and re-probed with antibodies against actin. Nearly equal amounts of actin were present in FP_A and FP_B cells throughout the time course in the blot. EGR-1 was found to be induced in an agonist-stimulated manner in FP_A cells and FP_B cells. This result was in agreement with the result from the Northern blot analysis.

2.4 Discussion

In the present study, we identified a large number of genes that were regulated in agonist-stimulated FP_A and FP_B cells using microarray technology. These significantly regulated genes have been categorized into several groups on the basis of their known functions (Fig. 2.1).

A major concern regarding the cDNA microarray technology is accuracy. However, we consider this study is a success with high accuracy. We based our judgment on 3 parameters. First, 70% of gene expression profile of 20 genes from Northern blotting analysis, a gold standard for gene expression at the RNA level, is in agreement with that from cDNA microarray analysis. Second, the functions of multiple genes identified in cDNA microarray are in agreement with the physiological functions of $PGF_{2\alpha}$. For example, EGR-1, fos-like antigen 1 and Jun-B are identified to be up-regulated in the

cDNA microarray analysis. These genes are members of the early response gene family, members of which exhibit de novo transcription shortly after stressful or mitogenic stimulations, and are important in wound healing and the inflammatory reaction (Braddock et al., 2001). Considering the important role of $PGF_{2\alpha}$ in wound healing and inflammation (Willoughby et al., 2000), it is not surprising to identify the early up-regulation of this class of genes in agonist-stimulated FP_A and FP_B cells. Third, some genes identified have been reported to be regulated by the activation of the FP receptor. For example, CTGF was reported to be induced upon the activation of the FP receptor (Liang et al., 2003).

This report is the first demonstration of gene regulation through the activation of the FP receptor at the genomic level. The success of this analysis provides the foundation for identifying the signaling pathways responsible for gene regulation by the activation of the FP receptors.

Table 2.1: Genes Up-regulated or Down-regulated in $PGF_{2\alpha}$ Stimulated FP_A or FP_B Cells

Functional Group	**Access No.	FP_A	FP_B
Transcription			
HIV-1 Tat interactive protein, 60 kDa	AA017042	N.C.*	-*
Zinc finger protein	AA033532	N.C.	+*
Runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	AA146826	N.C.	+
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1	AA432143	N.C.	+
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	AA451716	N.C.	+
Zinc finger protein 162	AA454673	N.C.	+
Ets variant gene 5 (ets-related molecule)	AA463830	+	+
Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	AA464856	N.C.	+
Murine leukemia viral (bmi-1) oncogene homolog	AA478036	N.C.	-
Immediate early response 3	AA480815	N.C.	+
Early growth response 1	AA486628	+	+
Nuclear receptor subfamily 4, group A, member 2	AA598611	N.C.	+
Transmembrane protein (63kD), endoplasmic reticulum/Golgi intermediate compartment	AA598787	N.C.	-
Kruppel-like factor 4 (gut)	H45711	+	+
Slug (chicken homolog), zinc finger protein	H57309	-	N.C.
Upstream binding transcription factor, RNA polymerase I	N92443	N.C.	-
Jun B proto-oncogene	N94468	N.C.	+
CAMP response element-binding protein CRE-Bpa	R21172	N.C.	+
Leucine-zipper-like transcriptional regulator, 1	R38194	N.C.	-
FOS-like antigen-1	T82817	N.C.	+
FOS-like antigen-1	T89996	N.C.	+
Jun B proto-oncogene	T99236	+	+
Sp3 transcription factor	W32135	N.C.	-
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	W55872	N.C.	+
V-myc avian myelocytomatosis viral oncogene homolog	W87741	N.C.	-
Signaling			
Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	AA070381	N.C.	+
KIAA0118 protein	AA076645	+	+
Janus kinase 1 (a protein tyrosine kinase)	AA284634	N.C.	-
GTP-binding protein overexpressed in skeletal muscle	AA418077	N.C.	+
Lymphocyte-specific protein tyrosine kinase	AA469965	-	N.C.
Tumor necrosis factor, alpha-induced protein 3	AA476272	N.C.	+
Protein kinase C binding protein 1	AA480906	N.C.	-
Transferrin receptor (p90, CD71)	AA488721	+	+
Insulin-like growth factor binding protein 3	AA598601	N.C.	+
Mitogen-activated protein kinase kinase 6	H07920	N.C.	-
Protein kinase H11	H57494	N.C.	+
KIAA0022 gene product	H60460	N.C.	-
Bromodomain-containing 2	H72520	N.C.	-
Protein tyrosine phosphatase, receptor type, A	H82419	N.C.	-
EphA2	H84481	+	+
RAB9, member RAS oncogene family	H98534	N.C.	+
Hyaluronan-mediated motility receptor (RHAMM)	R10284	N.C.	-
Myotubularin related protein 3	R10293	N.C.	+
Translocated promoter region (to activated MET oncogene)	R11490	+	+
Type I transmembrane protein Fn14	R33355	N.C.	+
Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3	R65928	N.C.	+
Arginine-rich, mutated in early stage tumors	R91550	+	+
Insulin-like growth factor 2 receptor	T62547	N.C.	+
Vav 1 oncogene	T65770	N.C.	+
Serine/threonine kinase 4	T94961	N.C.	+
Dual specificity phosphatase 5	W65461	+	+
Growth Factors and Interleukins			
Natural killer cell transcript 4	AA458965	N.C.	+
Thrombospondin 1	AA464630	-	N.C.
Connective tissue growth factor	AA598794	+	+

Interleukin 1, alpha	AA936768	N.C.	+
Delta (Drosophila)-like 1	R41685	-	N.C.
Interleukin 1 receptor antagonist	T72877	N.C.	+
Metabolism			
Spermidine/spermine N1-acetyltransferase	AA011215	N.C.	+
Enolase 2, (gamma, neuronal)	AA450189	N.C.	+
Phosphomevalonate kinase	H09914	N.C.	-
Carbonic anhydrase II	H23187	+	+
GS1999full	H25606	N.C.	-
Phytanoyl-CoA hydroxylase (Refsum disease)	N91990	N.C.	-
Cystathionase (cystathionine gamma-lyase)	R07167	+	N.C.
Prenylcysteine lyase	R78527	N.C.	-
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1	R82733	N.C.	+
Ferrochelatase (protoporphyria)	T64893	N.C.	-
Nicotinamide N-methyltransferase	T72235	N.C.	+
Oxysterol 7alpha-hydroxylase	W01800	N.C.	-
Translation			
Mitochondrial translational initiation factor 2	H18070	N.C.	-
Protein disulfide isomerase related protein (calcium-binding protein, intestinal-related)	N59626	N.C.	+
Tu translation elongation factor, mitochondrial	R45183	N.C.	-
Cytoskeleton			
Dynein, cytoplasmic, heavy polypeptide 1	AA410454	N.C.	+
H1 histone family, member 0	H57830	N.C.	-
Neurofilament, light polypeptide (68kD)	R14230	+	+
Cell Adhesion			
Protocadherin 20	AA040043	N.C.	+
Osteoblast specific factor 2 (fasciclin I-like)	AA598653	N.C.	+
Vascular cell adhesion molecule 1	H07071	N.C.	+
Cell Cycle			
Cyclin G1	AA083032	-	N.C.
Centromere protein E (312kD)	H94559	N.C.	-
Chromosome segregation 1 (yeast homolog)-like	N69204	N.C.	-
M-phase phosphoprotein 1	N91105	N.C.	-
Others			
KIAA1128 protein	AA114106	-	-
Tissue factor pathway inhibitor 2	AA399473	N.C.	+
No hit	AA405804	N.C.	-
Coagulation factor VIII, procoagulant component (hemophilia A)	AA437191	N.C.	+
No hit	AA442092	-	N.C.
Peroxisomal membrane protein 3 (35kD, Zellweger syndrome)	AA452566	+	+
ATP-binding cassette, sub-family B (MDR/TAP), member 1	AA455911	N.C.	+
V-myc avian myelocytomatosis viral oncogene homolog	AA464600	N.C.	-
No hit	AA488073	N.C.	+
N-myc downstream regulated	AA489261	N.C.	+
Thymine-DNA glycosylase	AA490546	N.C.	+
Splicing factor, arginine/serine-rich 5	AA598965	N.C.	-
Thioredoxin, mitochondrial	AI017377	N.C.	-
Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA-binding protein 1, 37kD)	H11069	N.C.	-
KIAA0449 protein	H14513	N.C.	+
Paternally expressed 10	H51765	+	+
No hit	H53268	N.C.	+
Hypothetical protein	H59188	N.C.	-
No hit	H59208	N.C.	-
No hit	H61003	N.C.	+
Hypothetical protein FLJ20392	H69785	N.C.	-
No hit	H70120	N.C.	-
No hit	H71857	N.C.	-
CASP8 associated protein 2	H50582	N.C.	-
Von Hippel-Lindau syndrome	H73054	N.C.	-
Chromosome 20 open reading frame 1	H73329	N.C.	-
Transmembrane protein 2	H75632	N.C.	+
No hit	H91256	N.C.	-
Nucleosome assembly protein 1-like 4	H92347	N.C.	-
High-mobility group (nonhistone chromosomal) protein 17	H93087	-	N.C.

No hit	H94947	N.C.	-
Cathepsin D (lysosomal aspartyl protease)	N20475	N.C.	-
V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	N20798	-	N.C.
KIAA1718 protein	N26802	N.C.	+
C-Myc target JPO1	N45440	N.C.	-
KIAA0914 gene product	N51424	N.C.	-
Hypothetical protein FLJ20008	N52394	N.C.	-
Hypothetical protein FLJ10099	N52973	N.C.	+
TJ6 protein	N70122	N.C.	+
No hit	N73575	N.C.	-
Fragile X mental retardation, autosomal homolog 1	N79708	N.C.	-
No hit	N91731	-	N.C.
No hit	N92035	N.C.	-
No hit	N94234	-	N.C.
No hit	R02654	N.C.	-
KIAA0648 protein	R02820	N.C.	-
Decay accelerating factor for complement (CD55, Cromer blood group system)	R09561	+	N.C.
No hit	R11605	N.C.	-
No hit	R25114	-	N.C.
No hit	R26789	-	N.C.
No hit	R33154	+	+
Syntaxin 11	R33851	N.C.	+
Hypothetical protein FLJ10357	R34205	N.C.	+
Hypothetical protein PRO1847	R45056	+	+
No hit	R63298	N.C.	-
CDA14	R64203	N.C.	-
No hit	R64660	-	N.C.
Leman coiled-coil protein	R66633	+	+
No hit	R67336	N.C.	+
KIAA1571 protein	R68133	N.C.	+
No hit	R70488	N.C.	-
A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	R76553	+	+
No hit	R77213	N.C.	-
ATP-binding cassette, sub-family B (MDR/TAP), member 10	R83875	N.C.	-
Hypothetical protein NUF2R	R92435	N.C.	-
No hit	R95851	N.C.	-
Membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	R98851	N.C.	-
No hit	R99526	+	+
No hit	T62100	N.C.	+
No hit	T67053	N.C.	-
No hit	T68336	N.C.	+
No hit	T70413	N.C.	-
Apolipoprotein C-IV	T71886	N.C.	+
No hit	T78110	N.C.	+
Coatomer protein complex, subunit alpha	T81091	+	+
No hit	T89283	N.C.	+
FK506-binding protein 8 (38kD)	W25035	N.C.	-
Caspase 6, apoptosis-related cysteine protease	W45688	N.C.	-
RNA binding motif protein 5	W73892	N.C.	-

*: N.C.: No change, +: Up-regulation, -: Down-regulation

**: Access no. represent ESTs. The corresponding gene were matched using blastn in the gene bank of NCBI.

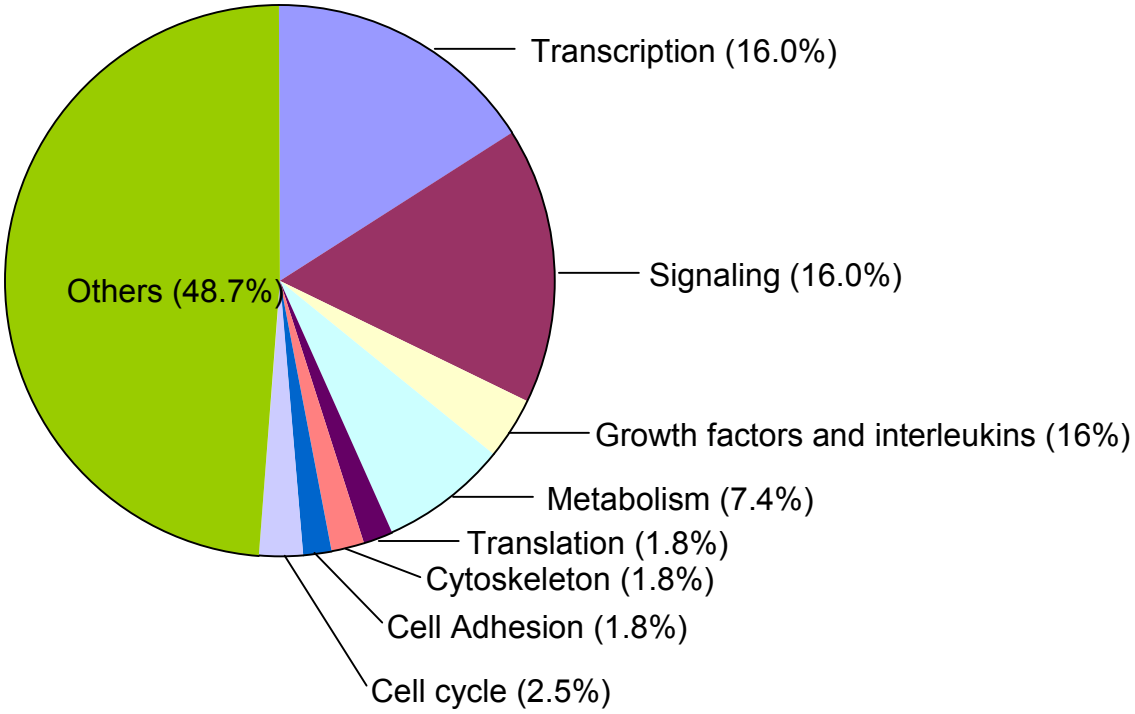

Fig.2.1 Piechart of functional grouping of the genes identified as down-regulated or up-regulated by the activation of FP receptor utilizing cDNA microarray analysis.

Table 2.2: Gene List for Northern Blot Analysis

Function Group	Access No.**	Microarray Results		Northern Results	
		FP_A	FP_B	FP_A	FP_B
Protocadherin 20*	AA040043	N.C.	+	++	+
Member of RAS oncogene family (RAB21)*	*AA076645*	+	+	+	++
Janus kinase 1	AA284634	N.C.	-	N.C.	+
MSG-1*	AA432143	N.C.	+	N.C.	+
EGR-1*	AA486628	+	+	+	++
N-myc Downstream Regulated*	AA489261	N.C.	+	+	++
Homo sapiens gene for T-cell nuclear receptor NOT (Nurr1), complete cds.*	AA598611	N.C.	+	+	++
Homo sapiens connective tissue growth factor (CTGF)*	AA598794	+	+	++	+
Interleukin 1, alpha	AA936768	N.C.	+	+	N.C.
EphA2*	H84481	+	+	+	++
C-myc target JPO1	N45440	N.C.	-	-	-
Protein Disulfide isomerase related protein*	N59626	N.C.	+	+	+
Homo sapiens matrix metalloprotease (ADAMTS1)*	R76553	+	+	+	N.C.
Arginin-rich, mutated in early stage tumors*	R91550	+	+	+	+
Vav 1 oncogene	T65770	N.C.	+	N.C.	N.C.
Interleukin 1 receptor antagonist	T72877	N.C.	+	N.C.	N.C.
FRA-1*	T82817	N.C.	+	+	++
FOS-like antigen-1*	T89996	N.C.	+	+	+
Jun-B*	T99236	+	+	+	++
FK506-binding protein 8 (38KD)	W25035	N.C.	-	N.C.	N.C.

N.C.: No change, +: Upregulation, -: Downregulation, ++: Robust upregulation
*: The Northern blots results are in agreement with microarray results.
**: Access no. represent ESTs. The corresponding gene were matched using blastn in the gene bank of NCBI.

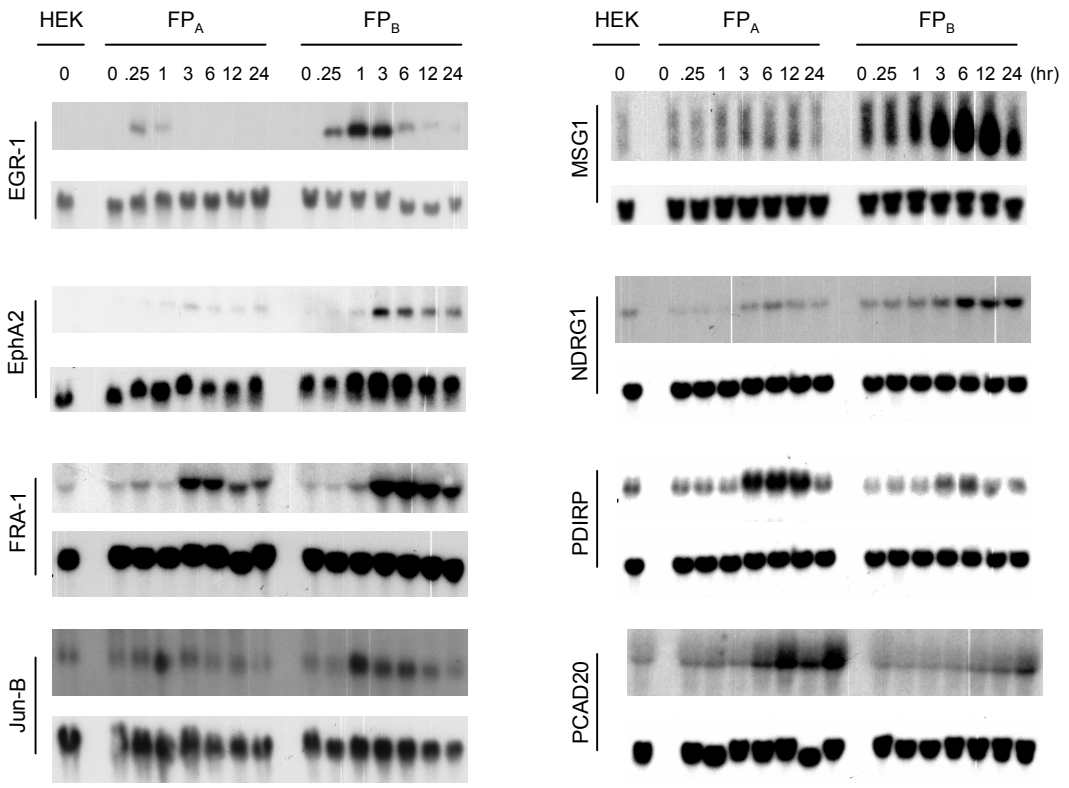

Fig. 2.2 Northern blots of genes that were found to be regulated in $PGF_{2\alpha}$ stimulated FP_A or FP_B-expressing cells. Cells were treated with 1 μM $PGF_{2\alpha}$ for the indicated times. RNA was extracted and subjected to Northern blot analysis as described in **Experimental Procedures**. Ten μg of total RNA was used for each lane. GAPDH, which was shown at the bottom blot for each gene, was used for loading control. Results are representative of at least two independent experiments. Abbreviations of gene names: EGR-1 for early growth response factor 1, EphA2 for ephrin A2, FRA-1 for fos related antigen 1, MSG-1 for melanocytes specific gene 1, NGF-β for neuron growth factor β, NDRG1 for N-myc downstream-regulated gene 1, PDIRP for protein disulfide isomerase related protein and PCAD20 for protocadherin 20.

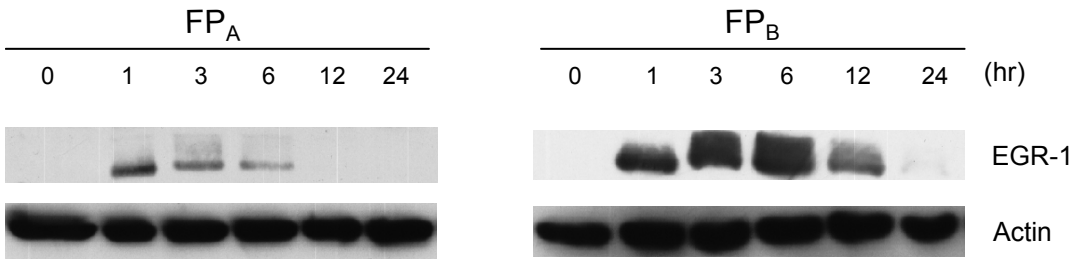

Fig. 2.3 Western blots of EGR-1 in FP_A or FP_B-expressing cells treated with $PGF_{2\alpha}$ for different lengths of time. Cells were treated with $PGF_{2\alpha}$ for the indicated time at 37^{o}C. The cells were then subjected to immunoblot as described in **Experimental Procedures**. Each blot was stripped and re-probed with antibodies against actin. Results are representative of three independent experiments.

CHAPTER THREE

CHARACTERIZATION OF THE SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF EGR-1 EXPRESSION BY THE ACTIVATION OF FP PROSTANOID RECEPTORS

3.1 Introduction

EGR-1 is a member of the zinc finger family of transcription factors. It is induced within 15-30 minutes after stimulation of fibroblasts and other cells with a variety of mitogenic stimuli. Expression of EGR-1 is related to suppression of cell growth, transformation and induction of apoptosis (Liu et al., 1998). Recently, EGR-1 has been related to heart hypertrophy (Khachigian et al., 2006; Buitrago et al., 2005). It was demonstrated that NAB1, a negative regulator of Egr-1, and Egr-1 serve as endogenous regulators of pathologic cardiac hypertrophy. Over-expression of NAB1 suppressed cardiomyocyte hypertrophy, skeletal organization, and protein synthesis, whereas cardiomyocyte growth was unaffected by a mutant form of NAB1 that lacked the Egr-binding site. Complementing these observations, ventricular and cardiomyocyte hypertrophy were reduced in Egr-1–null mice subjected to transverse aortic constriction-induced pressure overload.

Regarding the importance of EGR-1 in physiological process, the signal transduction pathways related to the gene regulation of EGR-1 have been extensively studied. Tyrosine kinase receptors have been related to the induction of EGR-1. For example, the induction of EGR-1 expression is induced by epithelial growth factor (EGF) (Tsai et al., 2000). GPCRs have also been reported to be involved in gene regulation of EGR-1. For example, the induction of EGR-1 in Swiss 3T3 fibroblasts is stimulated by PGE_2 (Danesch et al., 1994). For the downstream components of the receptors leading to the

induction of EGR-1, mitogen-activated protein kinase kinase ½ (MEK1/2) and its direct downstream component, extracellular signal-regulated kinase (ERK), are well known. For example, the induction of EGR-1 expression by lipopolysaccharide was shown to depend on the activation of MEK1/2 and ERK (Guha et al., 2001). The activation of the Ras/Raf pathway is necessary for the activation of MEK1/2. It has also been shown that Ras/Raf is involved in the induction of EGR-1 (Guha et al., 2001).

Similar signal transduction pathways, which were found to be involved in the gene regulation of EGR-1, were also identified to be through the FP receptor. For example, the activation of FP has been shown to activate Ras pathways (Sales et al., 2005). The activation of Raf kinase, a direct downstream component of Ras, has also been shown to occur through the FP receptor (Chen et al., 1998). Moreover, it has been shown that the activation of MEK1/2 is through the FP receptor (Sale et al., 2005).

In this study, we demonstrate that activation of the FP receptor can activate Ras, which turns on the Raf kinase, leading to the activation of MEK1/2 and induction of EGR-1. It is the first report to link the FP receptor with EGR-1. Considering the important role of FP (Adams et al., 1996) and EGR-1 (Khachigian et al., 2006; Buitrago et al., 2005) in heart hypertrophy, the induction of EGR-1 by the activation of FP may suggest a mechanism of FP induced heart hypertrophy.

3.2 Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, bovine serum albumin, hygromycin B, geneticin and gentamicin were obtained from Invitrogen (Carlsbad, CA). Anti-EGR-1 antibodies were obtained from Santa Cruz biotechnology (Santa Cruz, CA). Anti-rabbit IgG conjugated with horseradish peroxidase, vincullin antibody and all other unspecified chemicals were purchased from Sigma-Aldrich (St. Louis, MO). HRP-conjugated anti-Mouse IgG and nitrocellulose membrane were purchased from Bio-Rad (Hercules, CA). Cell lysis buffer was obtained from Cell Signaling (Boston, MA). $PGF_{2\alpha}$ was obtained from the Cayman Chemical Company (Ann Arbor, MI). Dominant negative C-Raf was provided by Dr. Morrison at NCI (Fradrick, MD) and Ras dominant negative was provided by Dr Vallancourt at University of Arizona (Tucson, AZ). PD 98059 was obtained from Calbiochem (San Diego, CA). Bay 43-9006 was provided by Dr. Hurley at University of Arizona (Tucson, AZ).

Cell Culture. FP cells were maintained at 37°C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 250 μg/ml geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin.

Western blotting. Cells were incubated at 37°C with 1 μM $PGF_{2\alpha}$ (Caymen Chemical) for 1 hour. In some cases cell were pretreated with either vehicle (0.1% Me_2SO or water) or 50 μM PD98059 (Calbiochem) for 30 minutes, or 20 μM Bay43-9006 for 1 hour at

37°C. In other cases, cells were transfected with C-Raf dominant negative or Ras dominant negative construct for 24 hours. Cells were scraped into 1x lysis buffer (10 x buffer from Cell Signaling) containing 1 μM PMSF and transferred to microfuge tubes. The samples were rotated for 16 hours at 4°C and were centrifuged at 16,000g for 15 minutes. Aliquots of the supernatants containing 50 μg protein were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad). Membranes were incubated in 5% non-fat milk for one hour at room temperature and were then incubated at 4°C for 16 hours with primary antibodies. The membranes were then washed three times and incubated with the corresponding secondary antibodies conjugated with a horseradish peroxidase in 5% non-fat milk at room temperature. After incubation with secondary antibody, the membranes were washed three times and immunoactivity was detected by SuperSignal (Pierce). The primary antibodies used were EGR-1 (Santa Cruz), phospho-MEK1/2 (Cell Signaling), phospho-ser 338 C-Raf (Cell Signaling), vincullin (Sigma), C-Raf (Santa Cruz) and MEK1/2 (Cell Signaling)

3.3 Results

MEK1/2 is involved in gene regulation of EGR-1 through the FP receptor. Because the activation of MEK1/2 is known to be necessary for the induction of EGR-1, we first checked to see if MEK1/2 is involved in the gene regulation of EGR-1 through the FP receptor. The dosage used for treatment was 1 μM and the time course was 1 hour. As shown in the upper panel of Figure 3.1 A, there was a phosphorylation of MEK1/2 in

HEK293 expressing human FP receptor (FP cells) treated with $PGF_{2\alpha}$. To check for an equal amount of MEK1/2 before and after $PGF_{2\alpha}$ treatment, the blot in the upper panel of Figure 3.1 A was re-probed with antibody against MEK1/2 protein. As shown in the lower panel of Fig. 3.1 A, the amount of MEK1/2 is nearly equal. In the same experiment, a specific MEK1/2 inhibitor PD-98059 was used to test if the activation of MEK1/2 is necessary for the induction of EGR-1 by activation of the FP receptor. The dosage of PD 98059 and $PGF_{2\alpha}$ was as described in **Experimental Procedures**. As shown in the upper panel of Figure 3.1 B, the induction of EGR-1 through the FP receptor was blocked by the pretreatment of PD-98059. To check the equal loading of protein lysates, the blot in the upper panel of Figure 3.1 B was re-probed with antibody against vincullin. As shown in the lower panel of Fig 3.1 B, an equal amount of vincullin suggests the equal loading of protein lysates in each lane. This experiment clearly shows that MEK1/2 is involved in the induction of EGR-1 by activation of the FP receptor.

C-Raf is involved in gene regulation of EGR-1 through the FP receptor. The well-known upstream component of MEK1/2 is Raf kinase. After finding that MEK1/2 is involved in the induction of EGR-1 through the FP receptors, we continued to examine whether Raf kinase is involved in the induction of EGR-1 through the FP receptor. At first, a Raf kinase specific inhibitor Bay43-9006 was used to examine if Raf kinase is involved in the induction of EGR-1. The dose and time course for treatment of Bay 43-9006 and $PGF_{2\alpha}$ were as described in **Experimental Procedures**. The upper panel of Fig 3.2A shows that Bay43-9006 inhibites the induction of EGR-1. This blot was re-probed

with antibody against vincullin and an equal amount of vincullin in the lower panel of Fig. 3.2A suggests the equal loading of protein lysates in each lane. B-Raf and C-Raf are the two main members of the Raf kinase family. However, Bay 43-9006 inhibits both kinases. To examine which kinase is involved in the induction of EGR-1, C-Raf and B-Raf dominant constructs were used. The transient transfection and drug treatment were as described in **Experimental Procedures**. As shown in the upper panel of Fig. 3.2 B, transient transfection of C-Raf dominant negative construct inhibits the induction of EGR-1 by the activation of FP receptor. The blot in the upper panel of Fig. 3.2 B was re-probed with antibody against vincullin and an equal amount of vincullin in the lower panel of Fig. 3.2 B suggests the equal loading of protein lysates in each lane. On the other hand, transient transfection of B-Raf dominant negative construct has no effect (data not shown). After C-Raf was identified to be involved in the induction of EGR-1, the activation of C-Raf through the FP receptor was examined. It was well documented that phosphorylation of Ser338 of C-Raf is related to the activation of C-Raf (Chong et al., 2001). Antibody against phospho-ser338 of C-Raf was used to detect the activation of C-Raf in FP_A cells treated with $PGF_{2\alpha}$. As shown in Fig. 3.2 C, the upper panel of Fig. 3.2 C shows phosphorylation of Ser338 in C-Raf after treatment with $PGF_{2\alpha}$. The blot in the upper panel of Fig. 3.2 C was re-probed with antibody against C-Raf and the result is presented in the lower panel of Fig. 3.2 C. The lower panel of Fig. 3.2 C shows that the amount of C-Raf is equal in each lane. In this experiment, C-Raf was found to be activated through the FP receptor and involved in the induction of EGR-1.

Ras is involved in gene regulation of EGR-1 through the FP receptor. After identification of the involvement of C-Raf in the induction of EGR-1 by activation of FP receptor, we tried to identify the upstream components of C-Raf pathways. The best-known upstream component is Ras small G protein. To test if Ras is involved in the induction of EGR-1, Ras dominant negative construct was used. The transient transfection and Western blot analysis were carried out as described in **Experimental Procedures**. In the upper panel of Fig. 3.3, the induction of EGR-1 by activation of the FP receptor is inhibited by the transfection of Ras dominant negative construct. For the control of equal loading, the blot in the upper panel of Fig. 3.3 was re-probed with antibody against vincullin. The result was shown in the lower panel of Fig. 3.3 and an equal amount of vincullin suggests the equal loading of protein lysates in each lane.

3.4 Discussion

$PGF_{2\alpha}$ has been reported to induce heart hypertrophy (Adams et al., 1996). However, little is known about the mechanism of this induction.

The receptor for $PGF_{2\alpha}$ is FP, which is a GPCR coupled with the Gq. It has been reported that the FP receptor can activate several small G proteins including Rho small G protein (Pierce et al., 1999) and Ras small G protein (Sales et al., 2006). In our study, Ras was also found to be activated through the FP receptor (Fig. 3.3). Interestingly, Ras has been shown to play an important role in heart hypertrophy (Proud et al., 2004).

The best-known direct downstream components of Ras are Raf kinases. Raf kinases include C-Raf, B-Raf and A-Raf. It has been reported that C-Raf and B-Raf, but not A-Raf can be activated by FP receptors (Chen et al., 1998). Raf kinase has also been shown to be involved in the heart hypertrophy (Proud et al., 2004). More specifically, C-Raf has been shown to play an important role in heart hypertrophy (Harris et al., 2004). In this study, transgenic mice expressing dominant negative C-Raf were found to be markedly resistant to the development of cardiac hypertrophy and hypertrophic gene induction in response to transverse aortic constriction. In our study, we also found that activation of C-Raf through the FP receptor, which leads to the induction of EGR-1 (Fig. 3.2).

MEK1/2 is the best-known downstream component of Raf kinases. MEK1/2 can be activated through the FP receptor (Sales et al., 2005) and has been reported to be involved in the heart hypertrophy (Xiao et al., 2001). In our study, we clearly show the activation of MEK1/2 through the FP receptor, which is necessary for the induction of EGR-1. One interesting aspect of our findings is the MEK1/2 independent activation of ERK1/2. In FP cells, ERK1/2 has been phosphorylated without agonist treatment (data not shown). Further work, such as transfection of ERK1/2 dominant negative constructs, is necessary to identify the role of ERK1/2 in the induction of EGR-1 through the FP receptors.

In the present study, we identified that the Ras/Raf/MEK1/2 pathways are involved in the induction of EGR-1 by activation of the FP receptor. Considering that both FP and EGR-1 play important roles in heart hypertrophy, this induction of EGR-1 by activation of the FP receptor may suggest a possible mechanism of FP-induced heart hypertrophy.

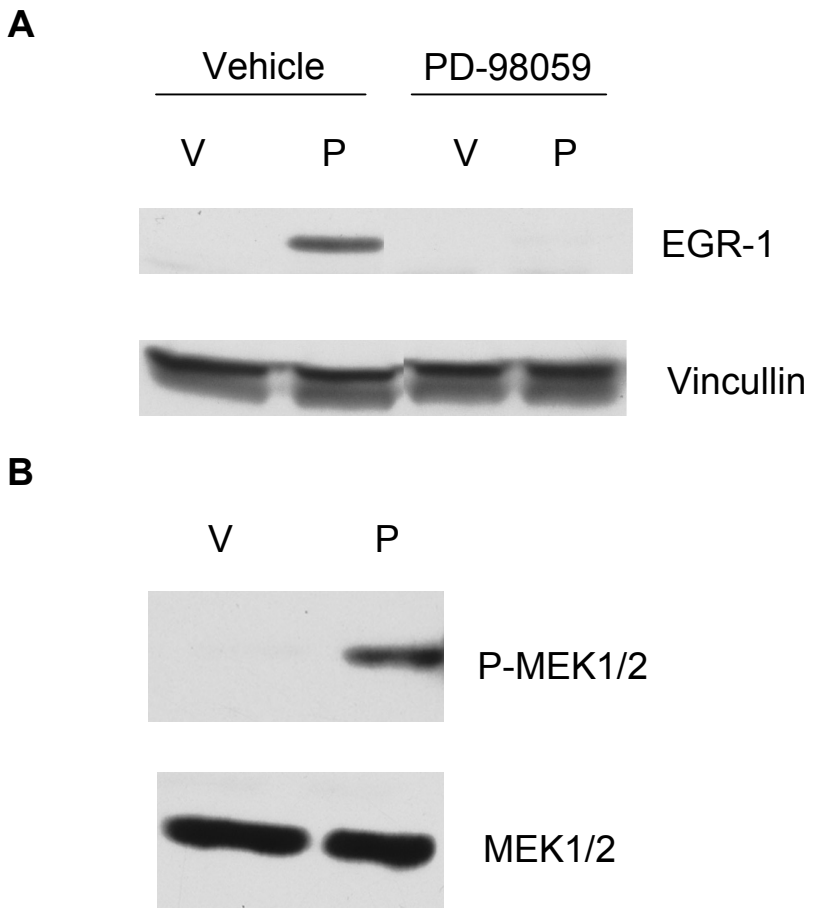

Fig. 3.1 MEK1/2 is involved in the gene regulation of EGR-1 through the FP receptor. A, human FP cells were pretreated with either vehicle or 50 μM PD-98059 for 1 hour after treatment with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 1 h at 37 °C and were then immediately subjected to immunoblot analysis as described under **Experimental Procedures.** In the upper panel, antibody against EGR-1 is used. The blots in the upper panel is re-probed with antibody against vincullin and the result is shown in the lower panels. **B**, human FP were treated with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 1 h at 37 °C and were then immediately subjected to immunoblot analysis as described under **Experimental Procedures.** In the upper panels, antibody against phospho MEK1/2 is used. The blots in the upper panels were re-probed with antibody against MEK1/2 and the results are shown in the lower panels. Immunoblotting results are representative of three experiments with each antibody and condition.

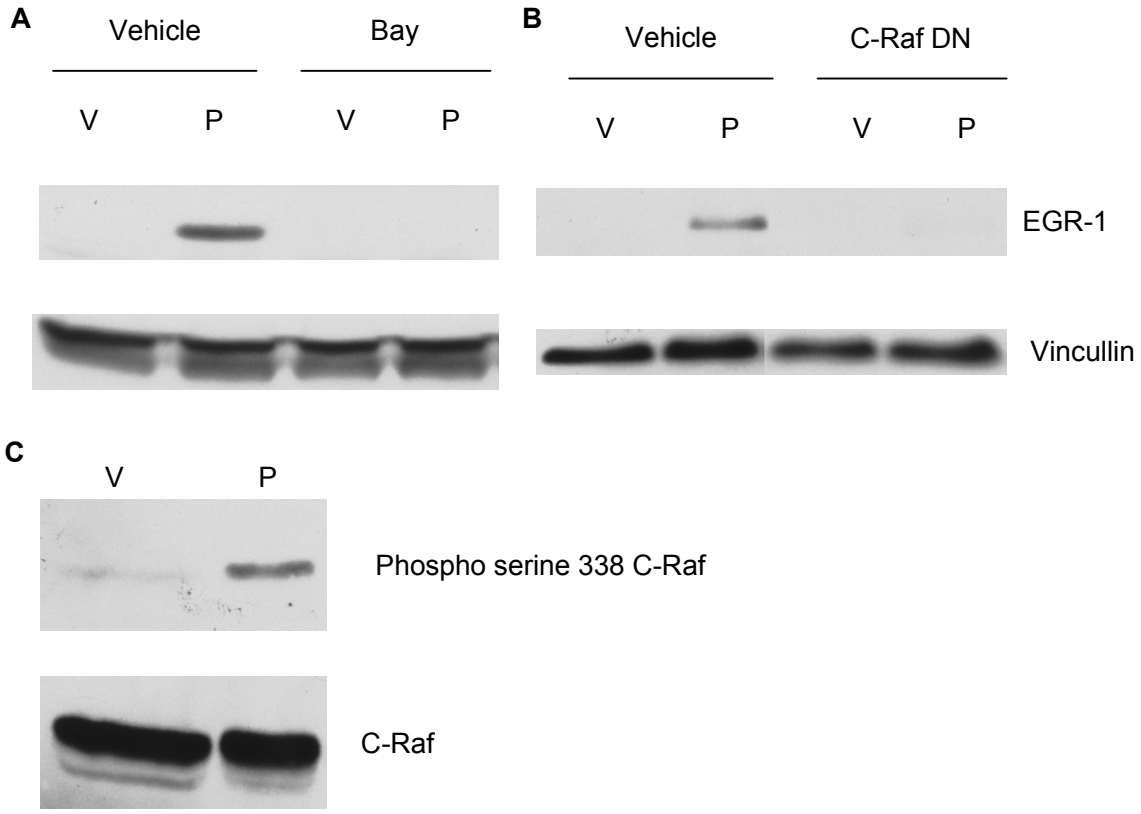

Fig 3.2 The C-Raf is involved in the induction of EGR-1 through the FP receptor. A and B, Human FP cells were treated with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis as described under **Experimental Procedures.** In some cases, the cells were pretreated with either vehicle, 20 μM Bay43-9006 for 1 hour (**A**). In other cases, the cells were transfected with vehicle or C-Raf dominant negative construct (C-Raf DN) for 16 hours (**B**) before adding $PGF_{2\alpha}$. In the upper panels, antibody against EGR-1 was used. The blots in the upper panels were re-probed with antibody against vincullin and the results are shown in the lower panels. **C**. human FP were treated with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 1 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures.** In the upper panel, antibody against phospho-ser 338 C-Raf is used. The blot in the upper panel was re-probed with antibody against C-Raf and the result is shown in the lower panels. Immunoblotting results are representative of three experiments with each antibody and condition.

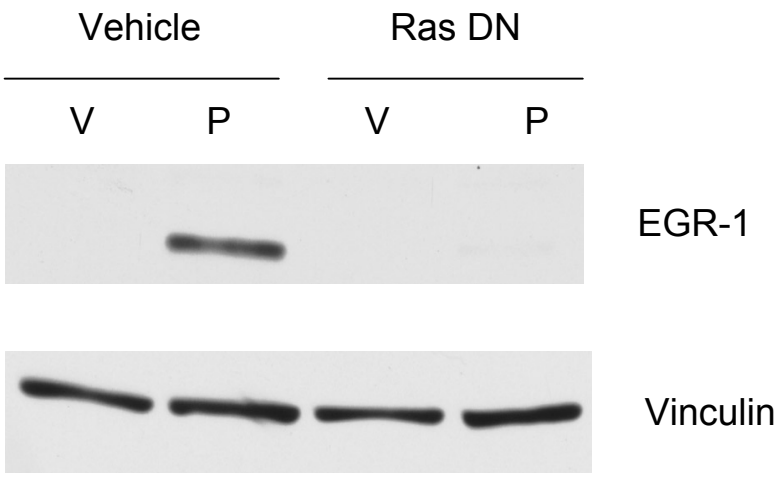

Fig 3.3 Ras is involved in gene regulation of EGR-1 through the FP receptor. Human FP cells were transfected with Ras dominant negative construct (Ras DN) followed by treatment with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 1 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures.** In the upper panels, antibody against EGR-1 was used. The blot in the upper panel was re-probed with antibody against vincullin and the results are shown in the lower panel. Immunoblotting results are representative of three experiments with each antibody and condition.

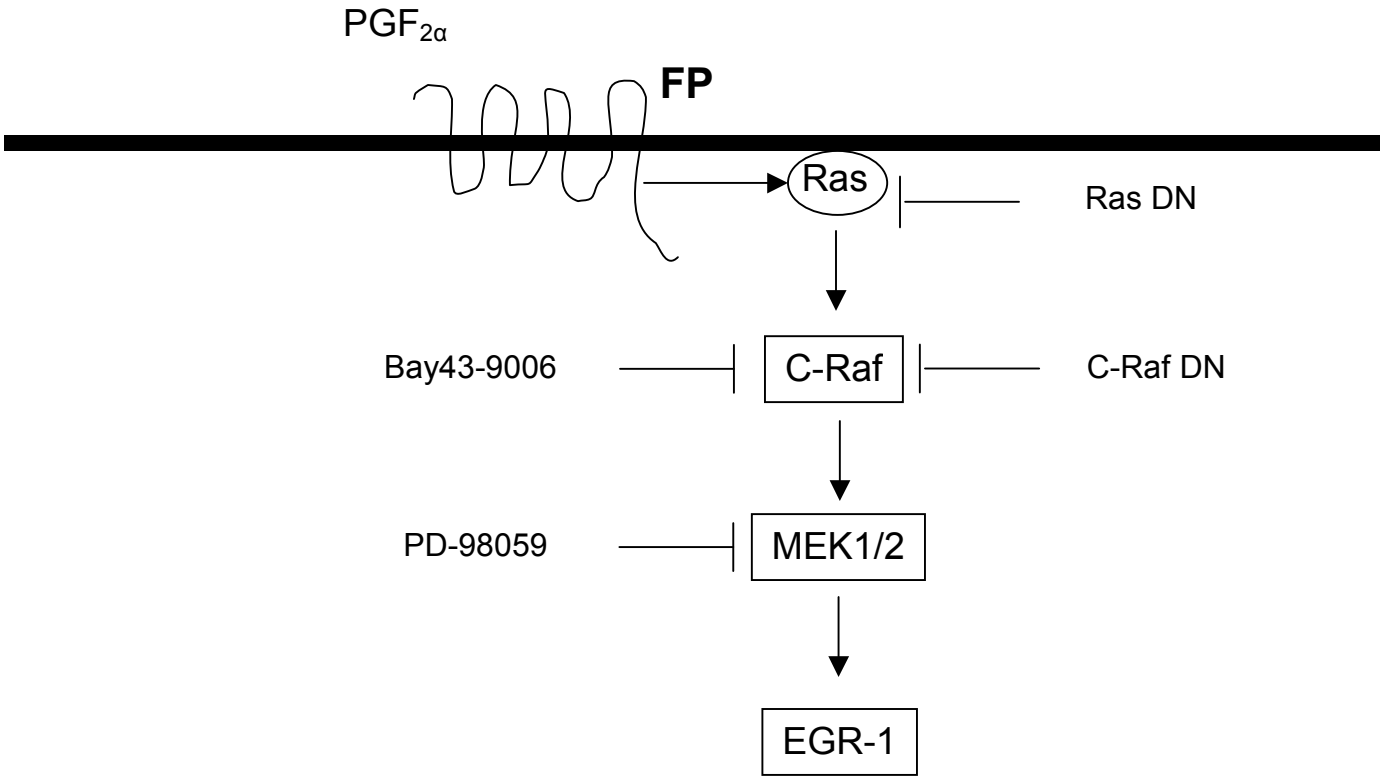

Fig 3.4 The signal transduction pathways involved in the induction of EGR-1 through the FP receptor. The FP receptor is capable of activating Ras, which in turn leads to the activation of C-Raf and MEK1/2. The activation of MEK1/2 induces the EGR-1, which may be necessary for the $PGF_{2\alpha}$-dependent heart hypertrophy.

CHAPTER FOUR

CHARACTERIZATION OF THE SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF CYR61 EXPRESSION BY THE ACTIVATION OF FP PROSTANOID RECEPTORS

4.1 **Introduction**

Cysteine-rich, angiogenic inducer, 61 (Cyr61) is a member of the CCN (Cyr61, CTGF, Nov) family. The CCN family is composed of matri-cellular regulatory factors involved in internal and external cell signalling. There are six members of the CCN family, namely Cyr61 (CCN1), CTGF (CCN2), CCN3, CCN4, CCN5 and CCN6 (Perbal et al., 2004). This family participates in angiogenesis, chondro-genesis, and osteogenesis and is probably involved in the control of cell proliferation and differentiation. The CCN family has been reported to play an important role in cancer. One example is that Cyr61 was shown to be involved in the breast cancer (Menendez et al, 2003). In this review, Cyr61 was shown to be involved in all aspects of breast cancer malignancy: angiogenesis, metasis, survival factor and cell proliferation. In addition to the breast cancer, Cyr61 is also shown to be involved in the invasion and angiogenesis of melanoma (Kunz et al., 2003).

Considering the important biological functions of Cyr61, some studies have been carried out to elucidate the signal transduction pathways related to the gene regulation of Cyr61. Tyrosine kinase receptors have been related to the induction of Cyr61. For example, Cyr61 expression is induced by epithelial growth factor (EGF) (Sampath et al., 2002). GPCRs have also been reported to be involved in the gene regulation of Cyr61. For example, Cyr61 is induced by activation of the FP receptor (Liang et al., 2003). For the downstream components of the receptors leading to the induction of Cyr61, multiple important signal transduction pathways have been identified as involved. One is the

Wnt/β-catenin pathway (Si et al., 2006), while another is small Rho small G protein (Liang et al., 2003). Raf kinase has also been involved in the induction of Cyr61 (Chung et al., 1998). Overall, however, the gene regulation of Cyr61 is not well established.

After identifying the induction of Cyr61 by activation of the FP receptor using Western blot analysis, we began to detail the signal transduction pathways leading to this induction. TCF/β-catenin has been shown to be activated through the FP receptor (Fujino et al, 2001). Raf kinase can also be activated through the FP receptor (Fig. 3.3). Interestingly, both pathways are involved in the gene regulation of Cyr61 (Si et al., 2006; Chung et al., 1998). Further study indicated that both TCF/β-catenin and B-Raf kinase are involved in the induction of Cyr61 through the FP receptor. Moreover, we found that activation of B-Raf is necessary for the activation of TCF/β-catenin in agonist stimulated FP cells. This is the first demonstration of crosstalk between the Raf kinase and TCF/β-catenin pathways. Considering the important roles of Raf kinase and TCF/β-catenin in cancer, these findings may have significant impact on cancer research.

4.2 Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, bovine serum albumin, hygromycin B, geneticin and gentamicin were obtained from Invitrogen (Carlsbad, CA). Anti-Cyr61 antibodies were obtained from Santa Cruz biotechnology (Santa Cruz, CA). Anti-rabbit IgG conjugated with horseradish peroxidase, anti-vincullin antibody and all other

unspecified chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cell lysis buffer was obtained from Cell Signaling (Boston, MA). $PGF_{2\alpha}$ was obtained from the Cayman Chemical Company (Ann Arbor, MI). TCF4 Dominant negative construct was provided by Dr. Frearon in University of Michigan (Ann Harbor, MI). B-Raf and C-Raf dominant negative constructs were provided by Dr. Morrison in NCI (Frederick, MD). Dominant negative Ras construct was provided by Dr. Vallancourt in University of Arizona (Tucson, AZ). Cytochalasin D and Toxin B were obtained from Calbiochem (San Diego, CA). Bay 43-9006 was provided by Dr. Hurley at University of Arizona (Tucson, AZ). TOPflash and FOPflash luciferase constructs were obtained from Promega (Madison, Wisconsin).

Cell culture. FP and FP_B cells were maintained at 37°C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 250 μg/ml geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin. Microgalia cells were maintained at 37°C with 10% CO_2/90% air in Ealge's minimum essential medium (MEM) containing 6 g/l glucose, 10% fetal calf serum (FCS), 105 U/l penicillin and 0.1 g/l streptomycin.

Western blotting. Cells were incubated at 37°C with 1 μM $PGF_{2\alpha}$ (Caymen Chemical) for 6 hour. In some cases cell were pretreated with either vehicle (0.1% Me_2SO or water), 20 μM Bay43-9006 for 1 hour or Toxin B 10ng/ml for 16 hours at 37°C. In other cases, cells were transfected with C-Raf, B-Raf or Ras dominant negative constructs for 16

hours using FuGENE-6 (Roche Molecular Biochemicals). Cells were scraped into 1x lysis buffer (10 x buffer from Cell Signaling) containing 1 μM PMSF and transferred to microfuge tubes. The samples were rotated for 16 hours at 4^{o}C and were centrifuged at 16,000g for 15 minutes. Aliquots of the supernatants containing 30 μg protein were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad). Membranes were incubated in 5% non-fat milk for 1 hour at room temperature and were then incubated at 4^{o}C for 16 hours with primary antibodies. Then, the membranes were washed three times and incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase in 5% non-fat milk at room temperature. After incubation with a secondary antibody, the membranes were washed three times and immunoactivity was detected by SuperSignal (Pierce). The primary antibodies used were vincullin (Sigma), Cyr61 (Santa Cruz).

Luciferase assay. Cells were split into 6 well plates and the next day were transiently transfected with either 1 μg/well of the wildtype Tcf/Lef reporter plasmid TOPflash or the mutant plasmid FOPflash. FOPflash differs from TOPflash by the mutation of its Tcf binding sites and serves to differentiate Tcf/β-catenin-mediated signaling from background (Upstate Biotechnology, Inc.). Then, cells were treated with 1 μM $PGF_{2\alpha}$ (Caymen Chemical) for 16 hours. In some cases cell were pretreated with either vehicle (0.1% Me_2SO or water), 10 μM Bay43-9006, 2 μM cytochalasin D for 1 hour or 10 μM AL8810 (Cayman) for 15 minutes at 37^{o}C. In other cases, cells were co-transfected with 1 μg/well of C-Raf, B-Raf or Ras dominant negative constructs. The treated cells were

rinsed twice with ice-cold phosphate-buffered saline and extracts were prepared using the Luciferase Assay System (Promega). Luciferase activity in the extracts (~1 μg protein/sample) was measured using a Turner TD-20/20 luminometer and was corrected for background by subtraction of FOPflash values from corresponding TOPflash values.

Phalloidin Staining. FP_B cells were grown for 2 days on 22-mm round glass coverslips (VWR Scientific) in 6-well dishes under subconfluent conditions. Cells were pretreated with 20 μM Bay43-9006 for 1 hour followed by treatment with $PGF_{2\alpha}$ for 1 hour. Then, cells were fixed for 15 min in freshly made 4% paraformaldehyde in 1× phosphate-buffered saline, quenched 3 times for 10 min in 0.1 M glycine, pH 7.4, and permeabilized for 15 min in 2× SSC (30 mM NaCl, 300 mM sodium citrate) containing 0.1% (v/v) Triton X-100 (Bio-Rad). Cells were preincubated in blocking buffer (2× SSC, 0.05% Triton X-100, 2% goat serum, 1% bovine serum albumin, 0.01% sodium azide) for 30 min. The cells were then incubated with 0.1 unit of Texas red isothiocyanate-conjugated phalloidin (Molecular Probes) in 50 μl of blocking buffer at room temperature for 1 h. The cells were briefly washed in 1× phosphate-buffered saline and mounted using p-phenylenediamine. The cells were viewed by immunofluorescence microscopy with an Olympus BH-2 microscope with a 40× oil objective through a Texas red isothiocyanate filter cube.

PCR reaction. The FP receptor expressed on microglia cells was determined by RT-PCR. Total RNA from microglia cells was isolated by using the RNeasy mini kit

(Qiagen) following the manufacturer's protocol with on-column DNase digestion. 1 µg of total RNA were reverse transcribed in a total volume of 20 µl including 200 units of SuperScript™ III Reverse Transcriptase (Invitrogen), 25 units of RNase inhibitor (Invitrogen), 0.5 µg of oligo$(dT)_{15}$ primer, 0.5 mM of each dNTPs, and 1x first-strand buffer provided by Invitrogen. The reaction will be incubated at 42 °C for 90 min. Aliquots of the RT products were subsequently used for PCR amplification. 10 µl of RT products was brought to a volume of 50 µl containing 1 mM $MgCl_2$, 0.12 mM of each dTNPs, 1 unit of Taq polymerase (Invitrogen), 0.5 µM of both the upstream and downstream PCR primers for specific prostanoid receptors, and 1x PCR buffer, provided by Invitrogen. Amplification was carried out with a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) after an initial denaturation at 94 °C for 3 min. This was followed by 35 cycles of PCR using the following temperature and time profile: denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension of 72 °C for 10 min. The following primer was used: FP-R sense 5'-ATG TCC ATG AAC AAT TCC AAA CAG CTA GTG-3', antisense 5'-GGT TTT GTG ACT CCA ATA CAC CGC TC-3'and GAPDH sense 5'-TGG GTG TGA ACC ATG AGA AG-3', GAPDH antisense 5'-TCT ACA TGG CAA CTG TGA GG-3'. The PCR products were visualized by electrophoresis on a 1.2% agarose gel in 0.5x TBE buffer after staining with 0.5 µg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were photographed. p-Cep4 containing FP plasmids was used as positive control while water was used as negative control.

4.3 Results

Both Raf kinase and TCF/β-catenin pathways are involved in the induction of Cyr61 through the FP receptor. The induction of Cyr61 at mRNA level by activation of the FP receptor has been reported (Liang et al, 2003). However, the induction of Cyr61 at the protein level through the FP receptor has not been reported. As shown in both Fig. 4.1 A and Fig. 4.1 B, we found that the induction of Cyr61 through the FP receptor happens at the protein level. After this finding, we began to identify the pathways leading to this induction. The Raf kinase pathway, which has been found to be activated through the FP receptor (Fig. 3.3), has been shown to be involved in the induction of Cyr61 (Chung et al., 1998). As shown in Fig. 4.1 A, a Raf kinase specific inhibitor Bay43-9006 inhibits the induction of Cyr61 through the FP receptor. TCF/β-catenin pathway has also been shown to be involved in the induction of Cyr61 by the activation of the FP receptor (Si et al., 2006). To test if TCF/β-catenin is involved in the induction of Cyr61 through the FP receptor, we used a TCF4 dominant negative construct. As shown in Fig. 4.2 B, the induction of Cyr61 in agonist stimulated human FP expressing cells is inhibited. To ensure equal loading of proteins, the blots shown in the upper panels were re-probed with antibodies to vincullin and, as shown in the lower panels similar amounts of vincullin were present throughout the treatments. These results suggest that both Raf kinase and TCF/β-catenin pathways are involved in the induction of Cyr61 through the FP receptor.

Raf kinase is involved in the activation of TCF/β-catenin pathway through the FP receptor. Both Raf kinase (Chen et al., 1998) and TCF/β-catenin (Fujino et al., 2001) have been shown to be activated through the FP receptor. Both pathways have also been shown to be involved in the induction of Cyr61 through the FP receptor (Fig 4.1). However, the relationship between Raf kinase and TCF/β-catenin has never been reported. We use specific Raf kinase inhibitor Bay43-9006, B-Raf and C-Raf dominant negative construct to address this question. As shown in the Fig 4.2, pretreatment with Bay43-9006 inhibits the activation of TCF/β-catenin by activation of the FP receptor. There are three isoforms of Raf kinase: A-Raf, B-Raf and C-Raf and Bay43-9006 can inhibit all of them. To identify the isoform responsible for this activation of TCF/β-catenin through the FP receptor, we transiently tranfected the human FP cells with B-Raf and C-Raf dominant negative constructs. A-Raf dominant negative was not used because it has been shown that A-Raf can not be activated through the FP receptor (Chen et al., 1998). As shown in Fig. 4.2 B, transient transfection of B-Raf dominant negative construct, can inhibit the activation of TCF/β-catenin through the FP receptor. However, C-Raf dominant negative increase the activation of TCF/β-catenin through the FP receptor (data not shown). These results suggest that Raf kinase, specifically B-Raf kinase, is involved in the activation of TCF/β-catenin through the FP receptor.

Ras is involved in the activation of TCF/β-catenin pathway and the induction of Cyr61 through the FP receptor. Because it is well-known that activation of Ras is necessary for the activation of Raf kinase, we began to identify the role of Ras in the

induction of Cyr61 and activation of TCF/β-catenin pathway through the FP receptor. Ras dominant negative construct was applied to address this question. As shown in the Fig. 4.3 A, transient transfection of Ras dominant negative construct inhibits the activation of TCF/β-catenin pathway through the FP receptor. Transient transfection of Ras dominant negative construct also inhibits the induction of Cyr61 through the FP receptor (Fig. 4.3 B). These results suggest that Ras is involved in the activation of TCF/β-catenin and induction of Cyr61 through the FP receptor.

Rho and actin stress fiber are involved in the activation of TCF/β-catenin pathway and induction of Cyr61 through the FP receptor. It has been reported in our laboratory that Rho small protein is involved in the activation of TCF/β-catenin through the FP receptor (Fujino et al., 2002). After identification of TCF/β-catenin involvement in the induction of Cyr61 (Fig 4.1 B), we used toxin B, a specific Rho inhibitor, to check if Rho is involved in the induction of Cyr61 through the FP receptor. As shown in Fig. 4.4 A, pretreatment of toxin B inhibits the induction of Cyr61 through the FP receptor and inhibits the basal expression of Cyr61. Rho has been shown to be necessary for the actin stress fiber formation through the FP receptor (Pierce et al., 1999). Because Rho has also been shown to be involved in the activation of TCF/β-catenin through the FP receptor (Fujino et al., 2002), we used an actin stress fiber disruptor, cytochalasin D, to find out if actin stress fiber is necessary for the activation of TCF/β-catenin. As shown in Fig. 4.4 B, cytochalsin D inhibits the activation of TCF/β-catenin through the FP receptor. These

results suggest that both Rho and actin stress fiber are involved in the activation of TCF/β-catenin pathway and induction of Cyr61 through the FP receptor.

Raf kinase is involved in the formation of actin stress fiber by the activation of the FP receptor. After identification of the involvement of actin stress fiber formation in activation of TCF/β-catenin through the FP receptor, a question was raised. What is the relationship between Raf and stress fiber formation? To address this question, we used Raf kinase specific inhibitor Bay43-9006. As shown in Fig. 4.5 D, Bay43-9006 inhibits the formation of stress fiber formation through FP receptor. This result suggests that Raf kinase is involved in the formation of actin stress fiber by the activation of the FP receptor.

Activation of TCF/β-catenin pathway through the FP receptor in an endogenous system. All the above experiments were carried out in FP cells, where the FP receptor is over-expressed. This raised the question of whether all the findings in FP cells are due to the artificial effects derived from the over-expressing FP receptor. To address this question, a cell line derived from human microglia cells (microglia cells) was used. For the first step, we checked the expression of the FP receptor in this cell line. As shown in the third lane of Fig. 4.6 A, mRNA of the FP receptor is present in the microglia cell line. In the first lane of Fig. 4.6 A, water was used as a template and no mRNA of the FP receptor presents. This result served as a negative control. In the second lane of Fig. 4.6 A, a plasmid containing FP cDNA was used as a template. The result served as a positive

control. After proving the presence of FP receptor in the microglia cells, we began to identify the signal transduction pathways by the activation of the FP receptor in this cell line. To examine if the activation of TCF/β-catenin through the FP receptor happens in microaglia cells, a FP receptor antagonist AL8810 was used. As shown in Fig. 4.6 B, TCF/β-catenin pathway is activated in microglia cells stimulated with $PGF_{2\alpha}$, but this activation is inhibited by the pretreatment of AL8810. We also used Raf kinase specific inhibitor Bay43-9006 and actin stress fiber disruptor cytochalasin D to pretreat the microglia cells followed by the treatment with $PGF_{2\alpha}$. As the result shows in Fig. 4.6 C, the activation of TCF/β-catenin through the FP receptor in the microglia cells was inhibited by both Bay43-9006 and cytochalasind D. These results suggest that both Raf-kinase and actin stress fiber are involved in the activation of TCF/β-catenin through a natural FP receptor.

4.4 Discussion

Cyr61 has been shown to play important roles in multiple cancers including breast cancer (Menendez et al, 2003) and melanoma (Kunz et al., 2003). However, the signal transduction pathways regulating the gene expression of Cyr61 have not been well established. In the present study, we dissected the signal transduction pathways leading to the gene regulation of Cyr61 through the FP receptors and found some novel signal transduction pathways.

Cyr61 has been reported to be regulated by TCF/β-catenin pathways (Si et al., 2006). In that study, Si et al. reported that Cyr61 could be induced by Wnt-3A in marrow mesenchymal stem cells. However, it is possible that Wnt-3A can induce Cyr61 through a calcium dependent non-canonical Wnt signaling (Miller et al., 1999). Herein, a TCF-4 dominant negative construct was used to successfully inhibit the induction of Cyr61 through the FP receptor. It is the first report which indicates that Cyr61 can be regulated through the canonical Wnt signaling pathway. Raf kinase has been shown to be involved in the gene regulation of Cyr61 (Chung et al., 1998). However, this gene regulation of Cyr61 shown in the paper is at the mRNA level. In our study, the Raf kinase specific inhibitor Bay43-9006 has been shown to inhibit the induction of Cyr61 through the FP receptor at the protein level (Fig. 4.1 A).

After identification of both Raf kinase and TCF/β-catenin in the gene regulation of Cyr61 through the FP receptor, we tried to clarify the relationship between the two pathways. We found that Raf kinase (Fig. 4.2 A), specifically B-Raf but not C-Raf (Fig. 4.2 B), is involved in the activation of TCF/β-catenin pathway through the FP receptor. To rule out the possible artificial effect stemming from the engineered cell line, we also checked this involvement of Raf kinase in the activation of TCF/β-catenin in a microglia cell line where FP is endogenously expressed (Fig 4.6). The results from the microglia cells are very similar to those obtained from FP cells. We also got similar results in HEK293 cells expressing ovine FP receptors (data not shown). All these data suggest that the crosstalk between B-Raf and TCF/β-catenin may be ubiquitous. This is the first report that

suggests the crosstalk between the Raf kinase and TCF/β-catenin pathways. Considering the important roles of TCF/β-catenin and B-Raf in cancer, this finding may be very important for the cancer research. In melanoma cases, for example, activating somatic mutations in the B-Raf protooncogene were discovered in more than 60% of cases (Dong et al., 2003; Davies et al., 2002). TCF/β-catenin has also been shown to play important roles in melanoma (Larue et al., 2006). The identification of the direct link between B-Raf pathway and TCF/β-catenin pathways may pave the way to a better understanding of the mechanism of melanoma and possible cancer therapy for this disease.

Ras is a well-known upstream component of the Raf kinase pathway. After finding the involvement of B-Raf in the activation of TCF/β-catenin pathways leading to the induction of Cyr61 through the FP receptor, we examined whether Ras is involved in this induction. As shown in Fig 4.3, Ras is necessary for the activation of TCF/β-catenin pathway and induction of Cyr61 through the FP receptor. TCF/β-catenin pathway has been shown to be activated by K-ras (Li et al., 2005). However, the involvement of wild-type Ras in the activation of TCF/β-catenin pathway has not been reported until now; we are the first one to report it. It is also the first report to show that Ras is necessary for the induction of Cyr61. Considering that both Cyr61 (Menendez et al., 2003) and Ras play important roles in breast cancer, it is not surprising to find the link between them.

The other small G proteins Rho have also been shown to be involved in the activation of TCF/β-catenin and induction of Cyr61 through the FP receptor (Fujino et al., 2002; Fig.

4.4). Although the involvement of Rho in the induction of Cyr61 through the FP receptor has been reported (Liang et al., 2003), ours is the first report to show this at the protein level. Rho is well-known to play very important roles in the regulation of the cellular cytoskeleton and has been shown to be involved in the formation of actin stress fiber by the activation of the FP receptor (Pierce et al., 1999). So we began to examine the relationship between the FP-stimulated actin stress fiber formation and TCF/β-catenin pathway. As shown in Fig 4.4 A, actin stress fiber formation is necessary for the activation of TCF/β-catenin through the FP receptor. We also found this activation in microglia cells where the FP receptor is naturally expressed (Fig. 4.6 C). Although the actin cytoskeleton has been implicated in the Wnt signaling (Akiyama et al., 2006), this is the first report that provides the direct link between actin cell skeleton and the activation of TCF/β-catenin. It is a very interesting finding because Rho is well-known to play very important roles in cancer, especially metasis (Fritz et al., 2006).

After identification of the involvements of both Ras/B-Raf and Rho/actin stress fiber pathways in the activation of TCF/β-catenin pathways and induction of Cyr61, we questioned the exact relationship between these two signal transduction pathways. To answer this question, we examined the effect of Raf kinase specific inhibitor Bay43-9006 on the FP-dependent actin stress fiber formation. As shown in the Fig. 4.5, Bay43-9006 inhibits this actin stress fiber formation. It has been shown that Rho/Rock pathways and Ras/B-Raf pathways converge to regulate the actin stress fiber formation (Prichard et al., 2004). We think it is quite possible that similar things happen in our system. Ras/B-Raf

pathways are activated through the FP receptor. In the same time, agonist stimulated FP activate Rho. These two pathways converge to induce the actin stress fiber formation and actin stress fiber leads to the activation of TCF/β-catenin pathway, which induces the expression of Cyr61. But because of the complicated crosstalks between Ras and Rho signal transduction pathways (Bar-Saji et al, 2000), other possibilities exist. More studies are needed to address this question.

In the present study, we found that the activation of the Ras/B-Raf pathway and Rho pathway through the FP receptor converge to induce the actin stress fiber formation. The actin stress fiber formation in turn activates the TCF/β-catenin pathway, which leads to the induction of Cyr61. Multiple novel cross-talks were identified. The first is the crosstalk between the B-Raf and TCF/β-catenin pathways. The second is the crosstalk between actin stress fiber formation and TCF/β-catenin. The third is the crosstalk between Ras and Cyr61. All the pathways involved in these cross-talks play important roles in cancer, and identification of these novel cross-talks may lead to a better understanding of cancer, especially with regard to the role of FP.

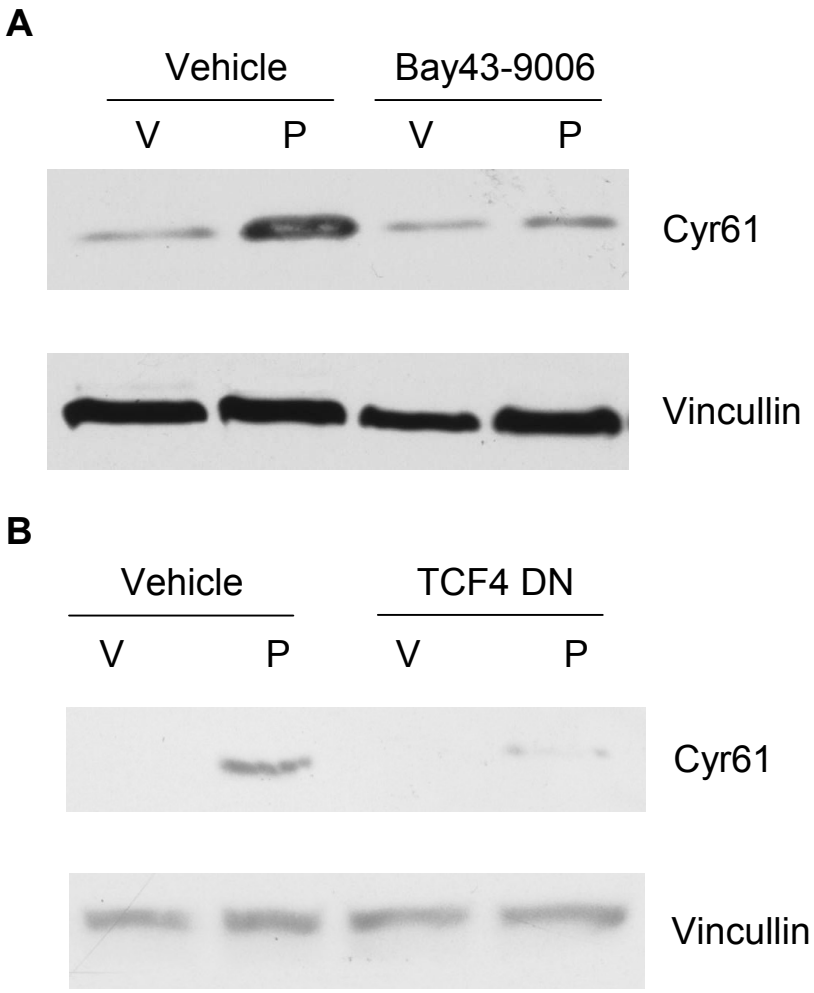

Fig. 4.1 Both Raf kinase and TCF/β-catenin pathways are involved in the induction of Cyr61 through the FP receptor. A, FP cells were pretreated with either vehicle or 20 μM Bay43-9006 for 1 hour followed by either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures**. B, FP cells were transfected with either vehicle or TCF4 dominant negative construct (TCF4 DN) for 16 hours followed by either vehicle (V) or $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures**. In the upper panels, antibody against Cyr61 is used. The blots in the upper panels are re-probed with antibody against vincullin and the results are shown in the lower panels. Immunoblotting results are representative of three experiments with each antibody and condition.

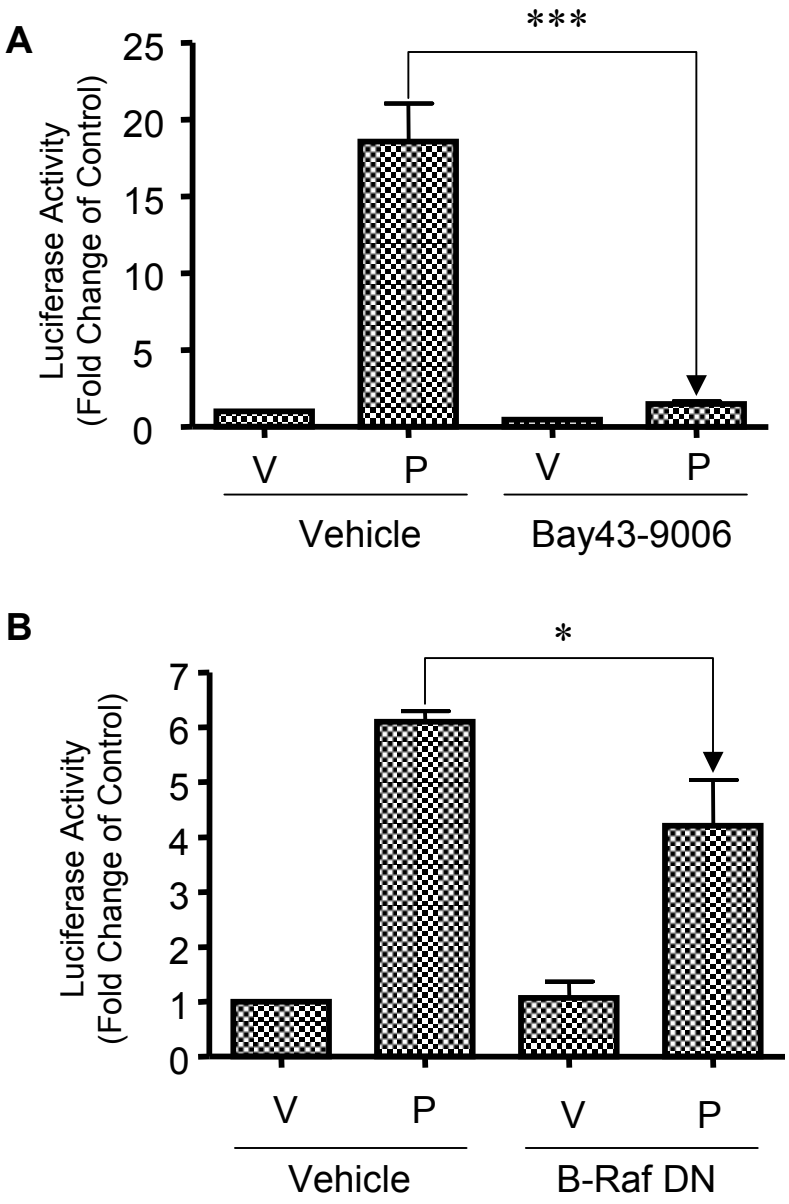

Fig 4.2 Raf kinase is involved in the activation of TCF/β-catenin pathway through the FP receptor. **A**. FP cells were transfected with FOPflash or TOPflash construct for 16 hours Then, FP cells were pretreated with either vehicle or 10 μM Bay43-9006 for 1 hour followed by treatment with $PGF_{2\alpha}$ for 16 hours. **B**. FP cells were co-transfected with TOFflash or TOPflash construct and B-Raf dominant negative construct (B-Raf DN) for 16 hours. Then cells were treated with either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 16 h. Shown is one representive experiment out of three independent experiments. Each point shown is the SEM of triplicate. Data are normalized to the vehicle-treated FP-expressing cells as 1. ***, $P<0.001$, as compared with vehicle-treated FP cells. *, $P<0.05$, as compared with vehicle transfected FP cells.

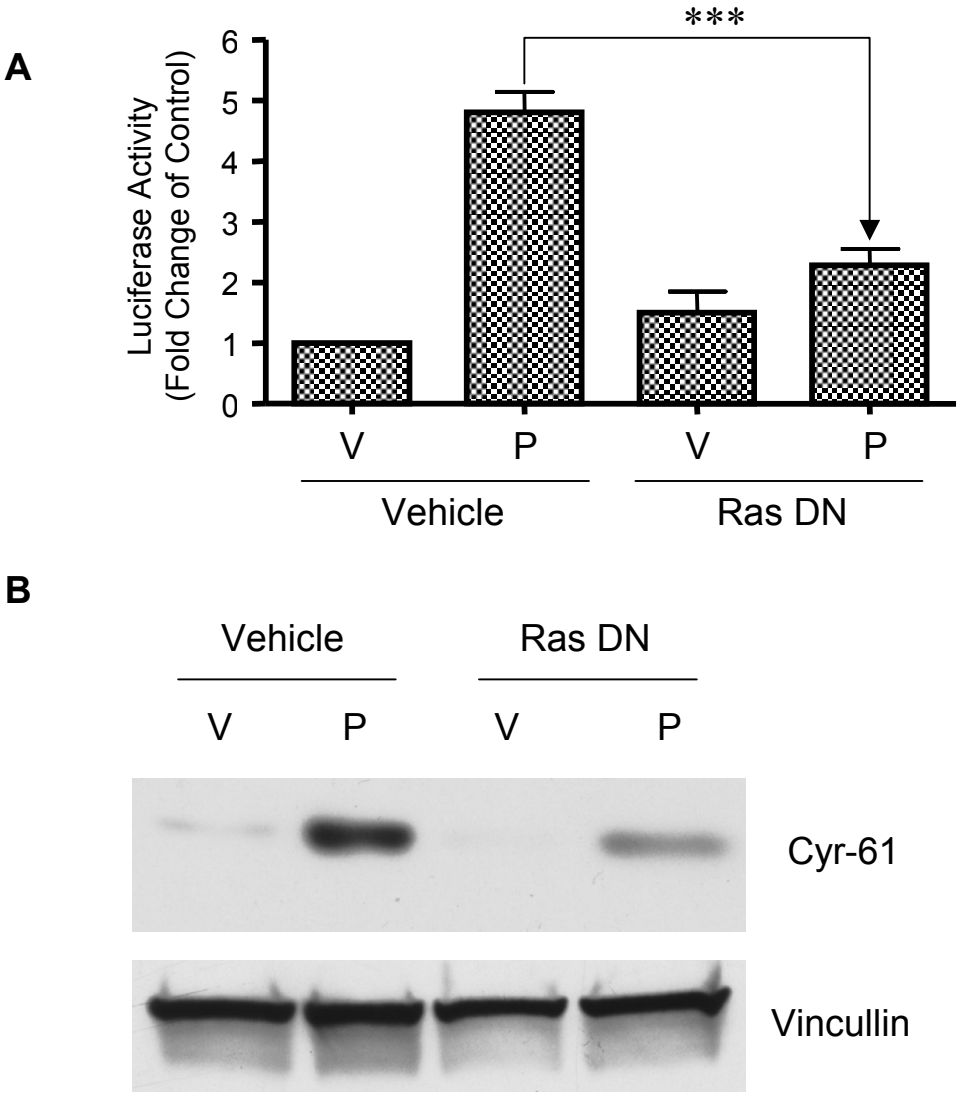

Fig 4.3 Ras is involved in the activation of TCF/β-catenin pathway and induction of Cyr61 through the FP receptor. A, FP cells were cotransfected with either TOPflash or FOPflash construct and Ras dominant negative construct (Ras DN) for 16 hours followed by treatment with either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 16 hours. Shown is one representive experiment out of three independent experiments. Each point shown is the SEM of triplicate. Data are normalized to vehicle-tranfected FP-expressing cells as 1. ***, $P<0.001$, as compared with vehicle-transfected FP cells. **B**, FP cells were transfected with either vehicle, or Ras dominant negative construct for 16 hours followed by either vehicle (V) or $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures**. In the upper panel, antibody against Cyr61 was used. The blot in the upper panel was re-probed with antibody against vincullin and the results were shown in the lower panels. Immunoblotting results are representative of three experiments with each antibody and condition.

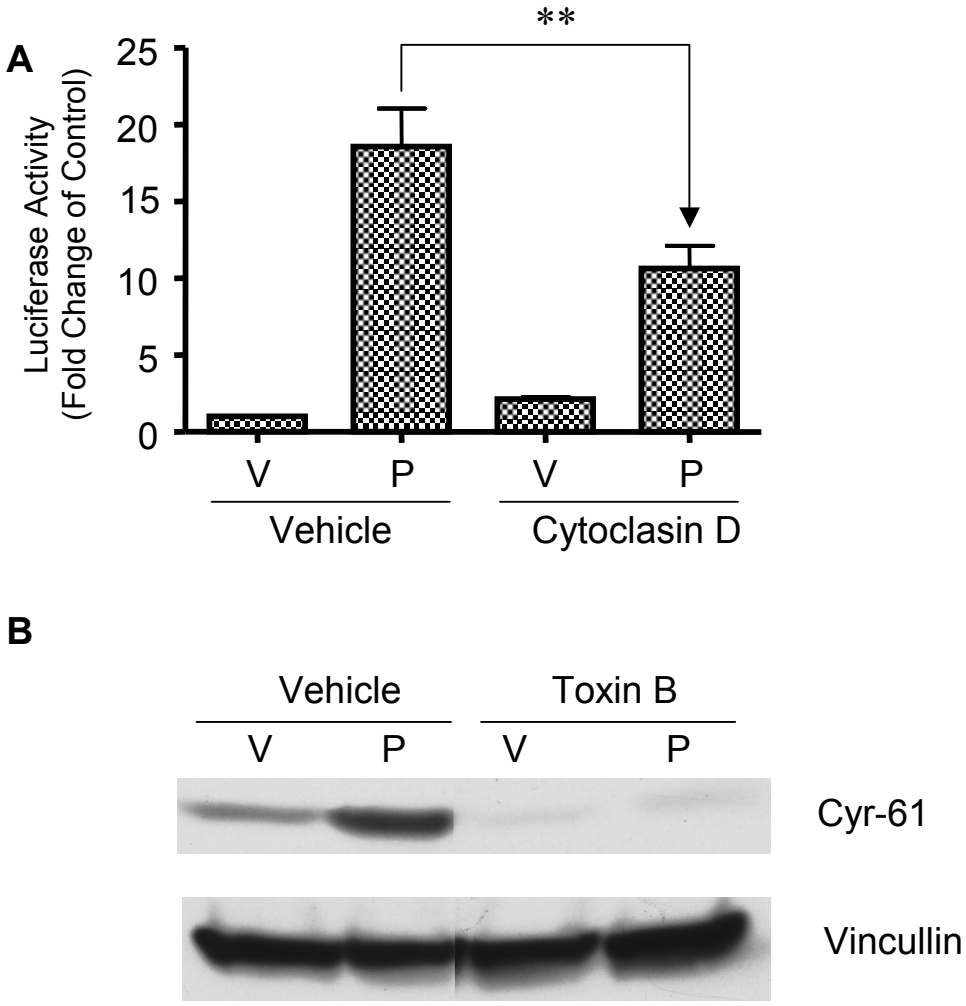

Fig 4.4 Rho and actin stress fiber is involved in the activation of TCF/β-catenin pathway and induction of Cyr61 through the FP receptor. A, FP cells were transfected with TOPflash or FOPflash construct. Then, cells were pretreated with either vehicle or 2 μM cytochalasin D for 1 hour followed by treatment with either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 16 hours. Shown is one representive experiment out of three independent experiments. Each point shown is the SEM of triplicate. Data are normalized to the vehicle-treated FP-expressing cells as 1. **, $P<0.01$, as compared with vehicle-treated FP cells. **B**, FP cells were pretreated with either vehicle, or 1 ng/ml for 16 hours followed by either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures.** In the upper panel, antibody against Cyr61 was used. The blots in the upper panels were re-probed with antibody against vincullin and the results are shown in the lower panel. Immunoblotting results are representative of three experiments with each antibody and condition.

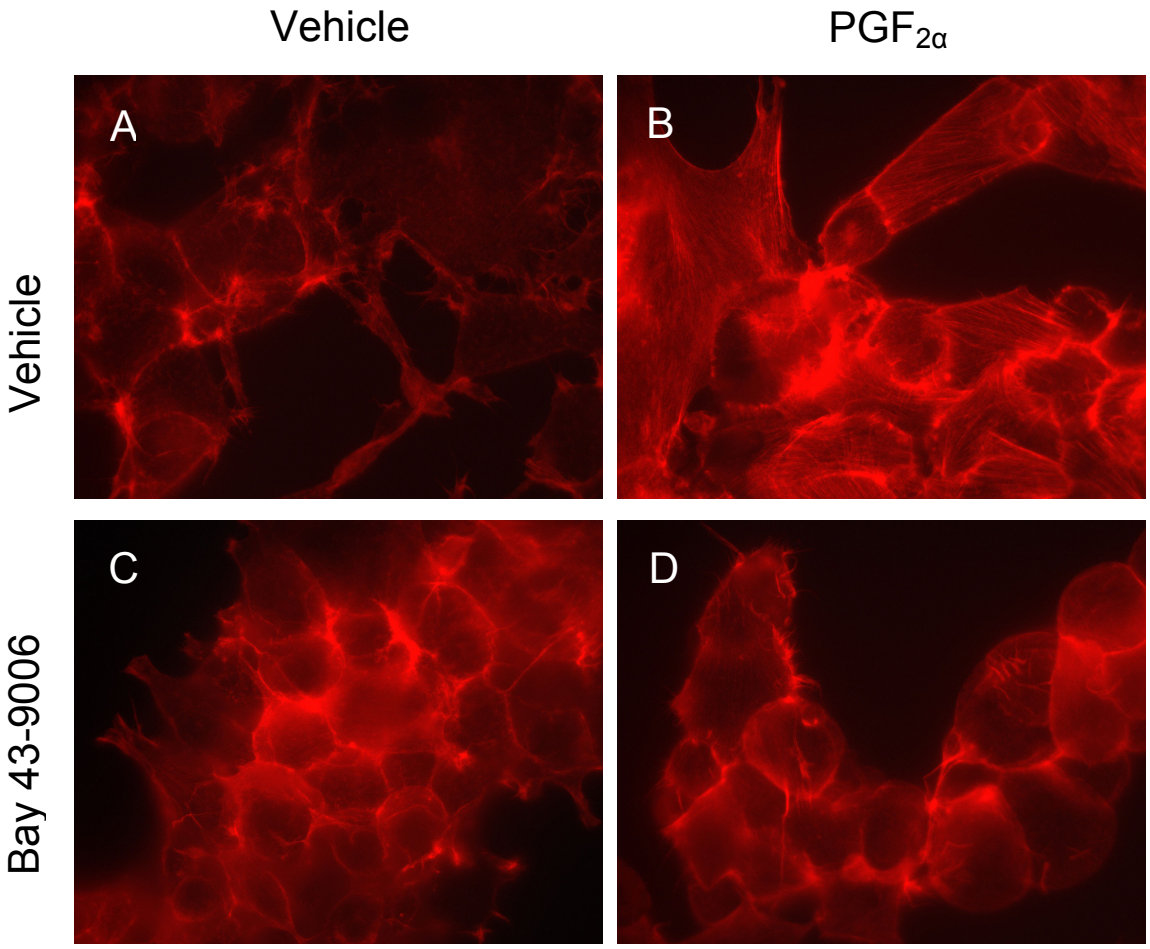

Fig. 4.5 Raf kinase induce the formation of actin stress fiber by the activation of FP receptor. FP_B cells were plated at low density on glass coverslips in 6-well dishes for 1 day. The cells were either not treated (panels A and B) or were pretreated with 20 μM Bay43-9006 for 1 h (panels C and D). The cells were then either untreated (panels A and C) or treated for 1 h with 1 μM $PGF_{2\alpha}$ (panels B and D). The cells were fixed and stained with phalloidin as described under **Experimental Procedures**. The images were obtained at 225×. These images are representative of three independent experiments.

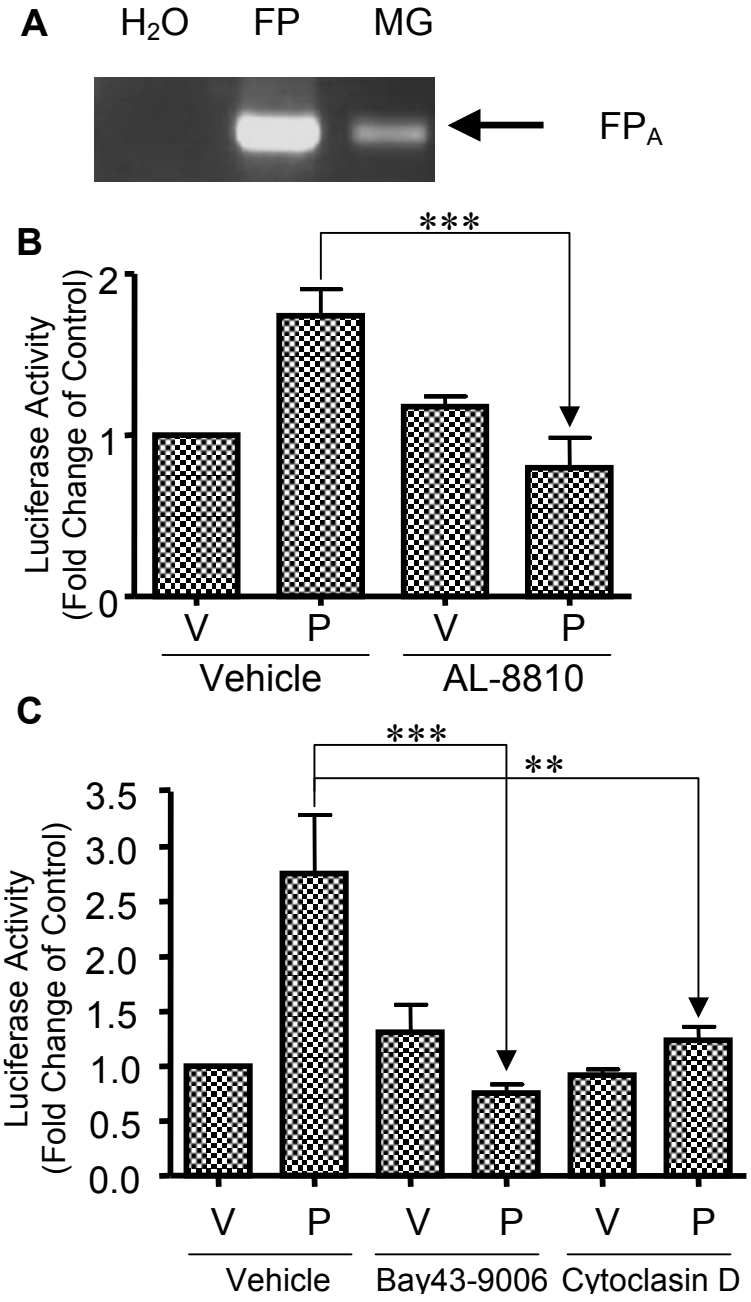

Fig. 4.6 Activation of TCF/β-catenin pathway through the FP receptor in an endogenous system. A, As described in **Experimental Procedures**, primers were designed to amplify FP receptor. Lane 1, corresponding negative control; Lane 2, a construct containing the human FP cDNA was used as template; lane 3, cDNA prepared from RNA extracted from human microglia cells. This result is representative of three independent experiments. **B**, Human microglia cells were pretreated with either vehicle or 10 μM AL-8810 for 15 minutes followed by treatment with either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 16 h. Shown is one representive experiment out of three independent experiments. Each point shown is the SEM of triplicate. Data are normalized to the vehicle-treated FP-expressing cells as 1. ***, $P<0.001$, as compared with vehicle-treated FP cells. **C**, Human microglia cells were pretreated with either vehicle , 10 μM Bay43-9006 or 2 μM cytochalasin D for 1 hour followed by treatment with either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 16 h. Shown is one representive experiment out of three independent experiments. Each point shown is the SEM of triplicate. Data are normalized to the vehicle-treated FP-expressing cells as 1. ***, $P<0.001$, as compared with vehicle-treated FP cells. **, $P<0.01$, as compared with vehicle-treated FP cells.

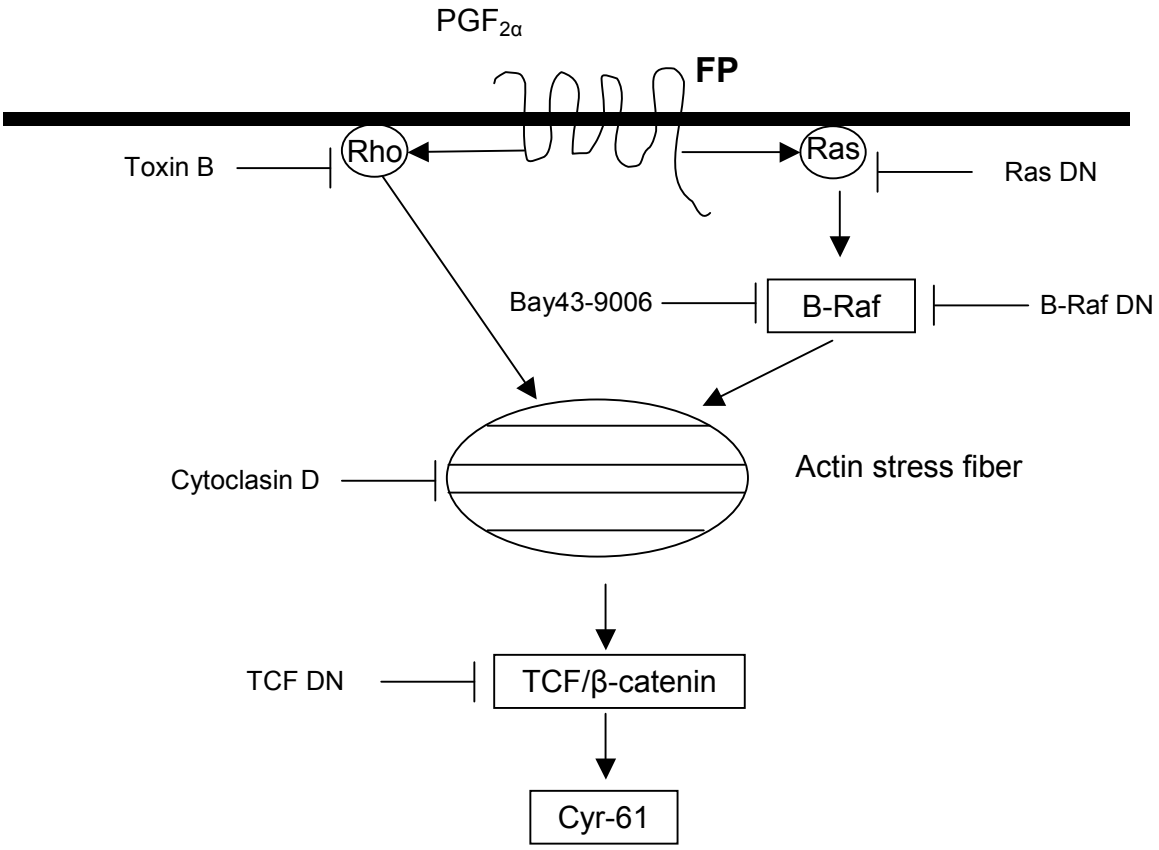

Fig 4.7 The signal transduction pathways involved in the induction of Cyr61 through the FP receptor. The FP receptor is capable of activating Ras, which in turn leads to the activation of B-Raf. Activated B-Raf converges with Rac/Rho pathway activated by the FP receptor to induce the formation of actin stress fiber. The activation of TCF/β-catenin leads to the induction of Cyr61.

CHAPTER FIVE

CHARACTERIZATION OF THE SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF CTGF EXPRESSION BY THE ACTIVATION OF FP PROSTANOID RECEPTORS

5.1 Introduction

Connective tissue growth factor (CTGF) is a member of the CCN (Cyr61, CTGF, Nov) family. CCN family is matri-cellular regulatory factors involved in internal and external cell signalling. There are six members of the CCN family, namely Cyr61 (CCN1), CTGF (CCN2), CCN3, CCN4, CCN5 and CCN6 (Perbal et al., 2004). This family participates in angiogenesis, chondro-genesis and osteogenesis, and is probably involved in the control of cell proliferation and differentiation. The CCN family has been reported to play important role in cancer. One example is that CTGF was shown to be over-expressed in pancreatic cancer, and antibodies against CTGF can attenuate tumor growth, metastasis and angiogenesis (Aikawa et al., 2006). Another example is the involvement of CTGF in the osteolytic metastasis of breast cancer (Shimo et al., 2006).

Considering the important biological functions of CTGF, some studies have been carried out to elucidate the signal transduction pathways related to the gene regulation of CTGF. Tyrosine kinase receptors have been related to the induction of CTGF. For example, CTGF is induced by TGF-β, whose receptor is tyrosine kinase receptor (Beddy et al., 2006). GPCRs have also been reported to be involved in the gene regulation of CTGF. For example, CTGF is induced by activation of the FP receptor (Liang et al., 2003). For the downstream components of the receptors leading to the induction of CTGF, multiple important signal transduction pathways have been implicated. One is the Wnt/β-catenin pathway (Luo et al., 2004). Ras has also been shown to be involved in the induction of CTGF (Phanish et al., 2005), as has another small G protein Rho (Liang et al., 2003).

More interestingly, hypoxia induced factor 1α (HIF-1α) has been reported to be necessary for the expression of CTGF (Haggins et al., 2004).

HIF-1α is a transcription factor identified to be induced in hypoxia condition (Semenza et al., 1992), which has been shown to play a very important role in tumors, especially solid tumors. One example is the over-expression of HIF-1α in most solid cancers (Zhong et al., 1999). Further study has indicated that HIF-1α increases the angiogenesis of cancer (Pugh et al., 2003). Because of the important functions of HIF-1α, much effort has been made to illuminate the gene regulation of HIF-1α. In the beginning, proteasomal related degradation was shown to play a major role in gene regulation of HIF-1α (Huang et al., 1998). Further study indicated that the heating shock protein 90 (HSP90) is involved in this degradation (Minet et al., 1999). On the other hand, multiple important signal transduction pathways, including Ras (Hirota et al., 2004), Raf (Lim et al., 2004), Rho (Hiyashi et al., 2005) and radical oxygen species (ROS) (Wang et al., 2004) have been shown to be involved in the gene regulation of HIF-1α. Recently, the induction of HIF-1α in normoxia conditions has been reported. One example is the PGE_2-stimulated induction of HIF-1α in prostate cancers (Liu et al., 2002).

In the present study, we confirmed the induction of CTGF by activation of the FP receptor at the protein level. After this, we began to detail the signal transduction pathways leading to this induction. We found that activation of Ras through the FP receptor can activate the Raf kinase pathway, which leads to the activation of TCF/β-

catenin. The activation of TCF/β-catenin leads to the induction of both HIF-1α and CTGF. This is the first demonstration that TCF/β-catenin is involved in the gene regulation of HIF-1α. We also found that receptor generated ROS is involved in the activation of TCF/β-catenin. This is the first demonstration that TCF/β-catenin can be activated through receptor generated ROS. Considering the important roles of HIF-1α and TCF/β-catenin in cancer, these findings may have significant impact on cancer research.

5.2 Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, bovine serum albumin, hygromycin B, geneticin and gentamicin were obtained from Invitrogen (Carlsbad, CA). Anti-CTGF antibodies and anti-goat IgG conjugated with horseradish peroxide were obtained from Santa Cruz biotechnology (Santa Cruz, CA). Anti-HIF-1α antibodies were obtained from BD bioscience (San Jose, CA). Anti-mouse IgG conjugated with horseradish peroxide was obtained from Promega (Madison, WI). Anti-rabbit IgG conjugated with horseradish peroxidase, PEG conjugated SOD (PEG-SOD), vincullin antibody and all other unspecified chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cell lysis buffer was obtained from Cell Signaling (Boston, MA). $PGF_{2\alpha}$ was obtained from Cayman Chemical Company (Ann Arbor, MI). Dominant negative TCF4 was provided by Dr. Frearon in University of Michigan (Ann Harbor, MI), Ras construct was provided by Dr. Vallancourt in University of Arizona (Tucson, AZ). 17-AAG, MG-132 and Toxin

B were obtained from Calbiochem (San Diego, CA). Bay 43-9006 was provided by Dr. Hurley at University of Arizona (Tucson, AZ). TOPflash and FOPflash luciferase construct were obtained from Promega (Madison, WI).

Cell culture. FP cells were maintained at 37°C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 250 μg/ml geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin. Microgalia cells were maintained at 37°C with 10% CO_2/ 90% air in Ealge's minimum essential medium (MEM) containing 6 g/l glucose, 10% fetal calf serum (FCS), 105 U/l penicillin and 0.1 g/l streptomycin.

Western blotting. Cells were incubated at 37°C with 1 μM $PGF_{2\alpha}$ (Caymen Chemical) for 6 hours. In some cases cell were pretreated with either vehicle (0.1% Me_2SO or water), 20 μM Bay43-9006 for 1 hour, Toxin B 10ng/ml for 24 hour, 93 μg/ml PEG-SOD for 15 minutes, 50 μM MG-132 for 1 hour, 2 μM 17-AAG for 24 hours or 10 μM AL8810 (Cayman) for 1 hour at 37°C. In other cases, cells were transfected with TCF4 or Ras dominant negative constructs for 16 hours. Cells were scraped into 1x lysis buffer (10 x buffer from Cell Signaling) containing 1 μM PMSF and transferred to microfuge tubes. The samples were rotated for 16 hours at 4°C and were centrifuged at 16,000g for 15 minutes. Aliquots of the supernatants containing 30 μg protein were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad). Membranes were incubated in 5% non-fat milk for one hour at room temperature and were then incubated at 4°C for 16 hours with primary antibodies. Then, the membranes

were washed three times and incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase in 5% non-fat milk at room temperature. After incubation with a secondary antibody, the membranes were washed three times and immunoactivity was detected by SuperSignal (Pierce). The primary antibodies used are vincullin (Sigma), CTGF (Santa Cruz) and HIF-1α (BD Bioscience).

Luciferase assay. Cells were split into 6 well plates and the next day were transiently transfected with either 1 μg/dish of the wildtype Tcf/Lef reporter plasmid TOPflash or the mutant plasmid FOPflash. FOPflash differs from TOPflash by the mutation of its Tcf binding sites and serves to differentiate Tcf/β-catenin-mediated signaling from background (Upstate Biotechnology, Inc.). Then, cells were pretreated with vehicle (1xPBS), or 93 μg/ml PEG-SOD (Sigma) for 15 minutes followed by treatment with 1 μM $PGF_{2\alpha}$ (Caymen Chemical) for 16 hours. The treated cells were rinsed twice with ice-cold phosphate-buffered saline and extracts were prepared using the Luciferase Assay System (Promega). Luciferase activity in the extracts (~1 μg protein/sample) was measured using a Turner TD-20/20 luminometer and was corrected for background by subtraction of FOPflash values from corresponding TOPflash values.

5.3 Results

The signal transduction pathways are involved in the induction of CTGF through the FP receptor. The induction of CTGF at the mRNA level by activation of FP receptor

has been reported (Liang et al., 2003). However, the induction of CTGF at the protein level through the FP receptor has not been reported. As shown in Fig. 5.1, we found that the induction of CTGF through the FP receptor happens at the protein level. After this finding, we began to identify the pathways leading to this induction. TCF/β-catenin pathway has also been shown to be involved in the induction of CTGF by the activation of FP receptor (Luo et al., 2004). As shown in Fig. 5.1 A, the induction of CTGF in agonist stimulated human FP expressing cells is inhibited by the transfection of TCF4 dominant negative construct. Rho small G protein has also been shown to be involved in the induction of CTGF through the FP receptor (Liang et al., 2003). As shown in Fig. 5.1 B, Rho inhibitor Toxin B inhibits the induction of CTGF through the FP receptor. Another small G protein Ras has been shown to be involved in the induction of CTGF (Phanish et al., 2005). As shown in Fig. 5.1 C, transfection of a Ras dominant negative construct inhibits the induction of CTGF through the FP receptor. After identification of Ras involvement in the induction of CTGF through the FP receptor, we also checked the role of Raf kinase, which is the direct downstream component of Ras, in this induction. As shown in Fig. 5.1 D, a Raf kinase specific inhibitor Bay43-9006 inhibits the induction of CTGF through the FP receptor. To ensure equal loading of proteins, the blots shown in the upper panels were re-probed with antibodies to vincullin, and as shown in the lower panels similar amounts of vincullin were present throughout the treatments. These results suggest that Ras, Rho, Raf kinase and TCF/β-catenin pathways are all involved in the induction of CTGF through the FP receptor.

The signal transduction pathways are involved in the induction of HIF-1α through the FP receptor. It has been reported that HIF-1α is necessary for the induction of CTGF (Haggins et al., 2004). So, we investigated whether HIF-1α is induced through the FP receptor. As shown in Fig. 5.2, we found that the induction of HIF-1α through the FP receptor happens at the protein level. After this finding, we began to identify the pathways leading to this induction. The Raf kinase pathway, which has been shown to be activated through the FP receptor (Chen et al., 1998), has been shown to be involved in the induction of HIF-1α (Lim et al., 2004). As presented in Fig. 5.2 A, a Raf kinase specific inhibitor Bay43-9006 inhibits the induction of HIF-1α through the FP receptor. After identification of Raf kinase involved in the induction of HIF-1α through the FP receptor, we examined whether the Raf kinase direct up-stream component Ras is involved in this induction. As shown in Fig. 5.2 B, transfection of a Ras dominant negative construct inhibits the induction of HIF-1α through the FP receptor. Another small G protein Rho has also been shown to be involved in the induction of HIF-1α (Hiyashi et al., 2005). As shown in Fig. 5.2 C, Rho inhibitor Toxin B inhibits the induction of HIF-1α in agonist stimulated FP cells. Because all the above signal transduction pathways have been shown to be involved in the activation of TCF/β-catenin through the FP receptors (Fig 4.2; Fig 4.3; Fujino et al., 2002), we hypothesized that TCF/β-catenin is involved in the induction of HIF-1α through the FP receptor. As shown in Fig. 5.2 D, transfection of TCF4 dominant negative construct inhibits the induction of HIF-1α through FP receptor. To ensure equal loading of proteins, the blots shown in the upper panels were re-probed with antibodies against vincullin, and as shown in the lower

panels, similar amounts of vincullin were present throughout the treatments. These results suggest that Ras, Raf kinase, Rho small G protein and TCF/β-catenin pathways are all involved in the induction of HIF-1α through the FP receptor.

Protein degradation plays a major role in the gene regulation of HIF-1α through the FP receptor. Proteosomal related degradation was shown to play a major role in gene regulation of HIF-1α (Huang et al., 1998). Further study indicated that HSP90 is involved in this degradation (Minet et al., 1999). After identification of induction of HIF-1α through the FP receptor, we examined whether the protein degradation is necessary for this gene regulation. As shown in Fig. 5.3 A, a proteosome inhibitor MG-132 clearly induces the expression of HIF-1α even without the stimulation of $PGF_{2\alpha}$. HSP90 has also been shown to be involved in the gene regulation of HIF-1α (Fig. 5.3 B). To ensure equal loading of proteins, the blots shown in the upper panels were re-probed with antibodies against vincullin, and as shown in the lower panels, similar amounts of vincullin were present throughout the treatments. These results suggest that protein degradation plays a major role in the gene regulation of HIF-1α through the FP receptor.

ROS is involved in the activation of TCF/β-catenin pathway and induction of HIF-1α through the FP receptor. ROS has been shown to be involved in the induction of HIF-1α (Wang et al., 2004). We therefore examined the role of ROS in the induction of HIF-1α through the FP receptor. As shown in Fig. 5.4 A, superoxide ion scavenger SOD inhibits the induction of HIF-1α through the FP receptor. The induction of CTGF, which is

regulated by HIF-1α, is also inhibited by SOD (Fig. 5.4 B). To ensure equal loading of proteins, the blots shown in the upper panels were re-probed with antibodies against vincullin, and as shown in the lower panels, similar amounts of vincullin were present throughout the treatments. Because TCF/β-catenin is involved in the gene regulation of HIF-1α (Fig. 5.2 D), we also examined whether ROS is involved in the activation of TCF/β-catenin. As shown in Fig. 5.4 C, SOD inhibits the activation of TCF/β-catenin pathway through the FP receptor. These results suggest that ROS is involved in the activation of TCF/β-catenin pathway, which leads to the induction of HIF-1α and CTGF through the FP receptor.

Regulation of HIF-1α through the FP receptor in an endogenous system. All of the above experiments were carried out in HEK293 cells over-expressing the human FP receptor. There is a concern that the same signal transduction pathways do not exist in a cell endogenously expressing FP receptor. To rule out this possibility, we chose human microglia cells that express the FP receptor (Fig. 4.6 A). As shown in Fig. 5.5, HIF-1α is induced in $PGF_{2\alpha}$ stimulated microglia cells, and this induction is inhibited by FP antagonist AL-8810. To ensure equal loading of proteins, the blot shown in the upper panel was re-probed with antibodies against vincullin, and as shown in the lower panel, similar amounts of vincullin were present throughout the treatments.

5.4 Discussion

CTGF has been shown to play important roles in multiple cancers (Aikawa et al., 2006). However, the signal transduction pathways regulating the gene expression of CTGF have not been well established. In the present study, we dissected the signal transduction pathways leading to the gene regulation of CTGF through the FP receptors and found some novel signal transduction pathways.

CTGF has been reported to be regulated by TCF/β-catenin pathways (Luo et al., 2004). In that study, Luo et al. reported the induction of CTGF in a cell line over-expressing β-catenin or Wnt-3A. However, it is possible that pathways other than the canonical Wnt pathway activated by the over-expression of β-catenin or Wnt-3A are involved in the induction of CTGF. Herein, a TCF-4 dominant negative construct was used and successfully inhibited the induction of CTGF through the FP receptor (Fig. 5.1 A). This is the first report to provide a direct link between CTGF and canonical Wnt signaling pathway. Because Rho small G protein is involved in the activation of TCF/β-catenin pathway through the FP receptor (Fujino et al., 2002), we also examine if Rho is involved in the induction of CTGF through the FP receptor. As shown in Fig. 5.1 B, Rho inhibitor Toxin B can inhibit the induction. This result corroborates the conclusion that TCF/β-catenin pathway is involved in the induction of CTGF through the FP receptor.

Ras has been shown to be involved in the gene regulation of CTGF (Phanish et al., 2005). We also identified that Ras is involved in the induction of CTGF through the FP receptor (Fig. 5.1 C). After this identification, we tried to establish the relationship between Raf

kinase and CTGF. This hypothesis is based on two points. First, Raf kinase is the direct downstream component of Ras. Second, Ras has been shown to be involved in the activation of TCF/β-catenin pathway through the FP receptor (Fig. 4.3), which is necessary for the induction of CTGF (Fig. 5.1 A). In our study, Raf kinase specific inhibitor Bay43-9006 has been shown to inhibit the induction of CTGF through the FP receptor at the protein level (Fig. 5.1 D). This is the first demonstration of the direct link between Raf kinase and CTGF.

HIF-1α was well-known to be involved in the induction of CTGF (Haggins et al., 2004). After identification of the induction of CTGF through the FP receptor, we examined if HIF-1α is involved in the induction of CTGF through the FP receptor. As shown in Fig. 5.2, HIF-1α is induced in agonist stimulated human FP cells. To rule out the possibility of artificial effect in HEK293 over-expressing FP receptor, we stimulated microglia cells, where FP is endogenously expressed, with $PGF_{2\alpha}$ and FP antagonist and got similar results (Fig. 5.5). This is the first report that HIF-1α can be induced by certain prostaglandin receptors. PGE2, a member of prostaglandin family, has been shown to induce the expression of HIF-1α (Liu et al., 2002). However, it is unknown which prostaglandin receptor is responsible for this induction because multiple PGE2 receptors express in the cell line used. After finding the induction of HIF-1α through the FP receptor, we examined whether the signal transduction pathways involved in the induction of CTGF are involved in the induction of HIF-1α by the activation of FP receptor. The first is the TCF/β-catenin pathway. As shown in the Fig 5.2 A, transfection

of TCF4 dominant negative construct inhibits the induction of HIF-1α through the FP receptor. This is the first report that TCF/β-catenin pathway is necessary for the induction of HIF-1α. Both pathways are very important in cancer, especially colon cancer. The discovery of this crosstalk may lead to a better understanding of cancer, maybe a novel drug target for the cancer therapy. More interestingly, it is recently reported that HIF-1α can interact with β-catenin and inhibit the transcription activity of β-catenin/TCF complex (Kaiti et al., 2007). This may indicate the complicated crosstalks between these two pathways, and further studies are necessary to address this question. After the identification of the involvement of TCF/β-catenin pathways in the induction of HIF-1α, we also examined the involvement of the pathways necessary for TCF/β-catenin through the FP receptor in the induction of HIF-1α. As shown in Fig. 5.2 B, Rho small G protein, which has been shown to be necessary for the activation of TCF/β-catenin pathway through the FP receptor (Fujino et al., 2002), is involved in this induction. This result, which is similar to previous findings (de Gooyer et al., 2006), further corroborates that TCF/β-catenin is necessary for the induction of HIF-1α. Ras/Raf pathways, which have been also shown to be necessary for induction of TCF/ β-catenin (Fig. 4.3; Fig. 4.4), have also been shown to be involved in the induction of HIF-1α through the FP receptor (Fig. 5.2 C; Fig. 5.2 D). These results also corroborate that TCF/β-catenin is necessary for the induction of HIF-1α through the FP receptor.

Another signal transduction pathway involved in the induction of HIF-1α is ROS (Wang et al., 2004). Recently, increasing evidence suggests that ROS, generated by membrane

NADPH oxidase (NOX), can work as signal transduction molecules. Moreover, activation of angiotensin II receptor, a Gq coupled receptor, can generate ROS leading to the induction of MCP-1 (Tanifiji et al., 2005). So after identification of induction of HIF-1α through the FP receptor, which is also a Gq receptor, we examined whether ROS is involved in this induction. As shown in Fig. 5.4 A, SOD, a scavenger of superoxide ion, inhibits the induction of HIF-1α through the FP receptor. SOD also inhibits the induction of CTGF, which is regulated by HIF-1α, through the FP receptor (Fig. 5.4 B). Because TCF/β-catenin has been shown to be involved in the induction of HIF-1α through the FP receptor (Fig. 5.2 A), we examined if ROS is also involved in the activation of TCF/β-catenin through the FP receptor. As shown in Fig. 5.4 C, SOD inhibits the activation of TCF/β-catenin through the FP receptor. It has been reported that ROS can activate the TCF/β-catenin (Futano et al., 2006). However, a concern arises in this paper. Hydrogen peroxide at 50 mM was used to stimulate the TCF/β-catenin pathway, but hydrogen peroxide at this concentration may be toxic to the cells (Iannone et al., 1993). It is possible that hydrogen peroxide activates TCF/β-catenin through secondary toxic effect caused by hydrogen peroxide, rather than directly from ROS. However in our study, we demonstrated in the first time that ROS, possibly superoxide ion generated from a nature receptor, can activate the TCF/β-catenin pathway.

After identification of the role of ROS and NOX in the activation of TCF/β-catenin pathway through the FP receptor, a question was raised about the relationship of ROS with the other signal transduction pathways involved in the activation of TCF/β-catenin

pathway. It has been reported that Rac, a member of Rho subfamily, is necessary for the activation of NOX (Takeya et al., 2006). It was reported that Rho protein is involved in the activation of TCF/β-catenin through the FP receptor (Fujino et al., 2002) and this pathway may converge with Ras/B-Raf pathway to regulate the actin stress fiber formation and lead to the activation of TCF/β-catenin pathway through the FP receptor (Fig 4.7). Moreover, Rac protein has also been reported to be upstream of Rho, and it is necessary for the actin stress fiber formation (Guo et al., 2006; Kjoller et al., 1999). However, Rac induced ROS was shown not to be involved in the actin stress fiber formation (van Wetering et al., 2002). It is quite possible that activation of Rac stimulates NOX and generates ROS; at the same time, Rac activates Rho, which in turn converges with Ras/B-Raf pathways to induce actin stress fiber formation. Ros converging with actin stress fiber formation activate TCF/β-catenin pathway.

In the present study, we found that the agonist stimulated FP activates Ras pathway, which stimulates Raf-kinase. In the same time, agonist stimulated FP activates Rho subfamily, probably Rac, to stimulate the NOX and generate ROS. These two pathways converge to induce the stress fiber formation, which leads to the induction of activation of the TCF/β-catenin pathway. The activation of TCF/β-catenin pathway induces the expression of HIF-1α and CTGF. All these are summarized in the Fig 5.6. During the dissection of these pathways, two novel cross-talks between pathways were identified. The first is the involvement of TCF/β-catenin pathway in the induction of HIF-1α. The other is the activation of TCF/β-catenin by ROS generated from the activation of FP

receptor. Considering the important roles of these pathways in cancer, the identification of these cross-talks may lead to a better understanding of the role of FP receptor in cancer.

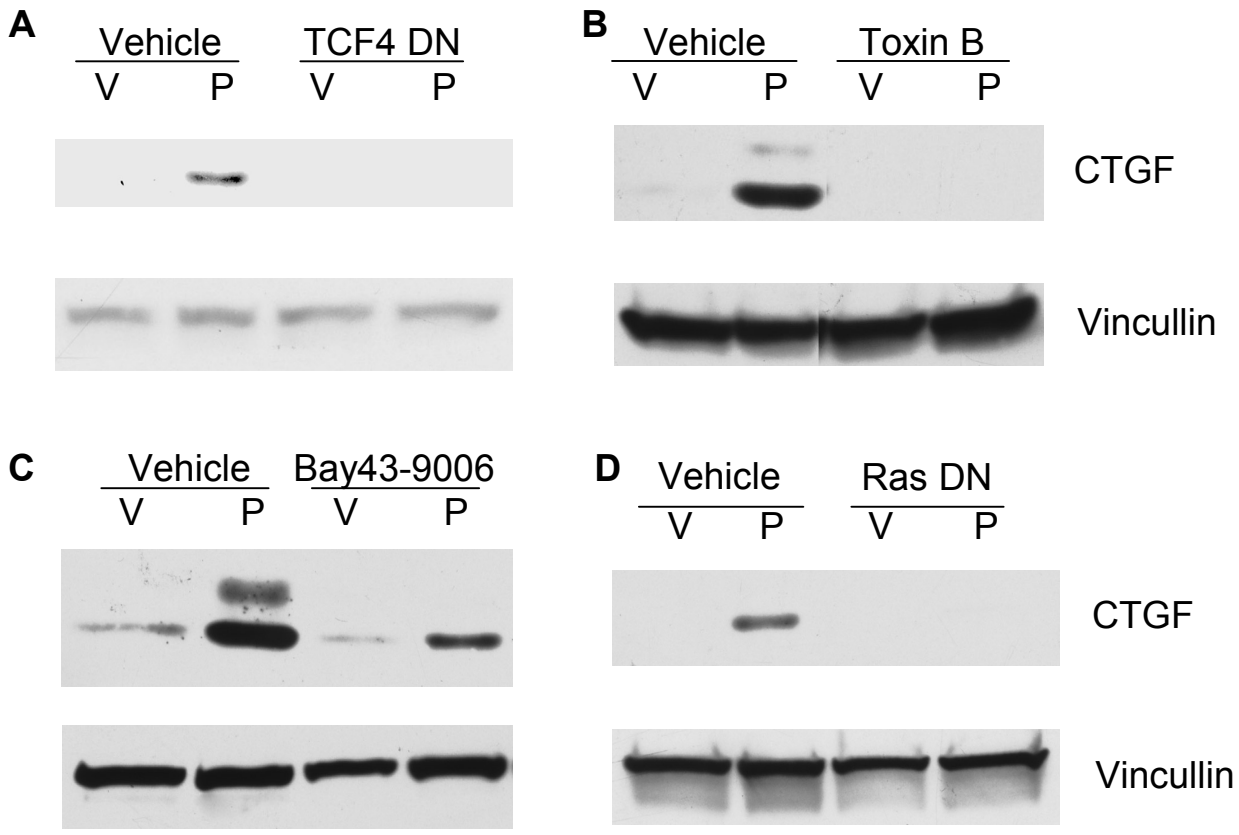

Fig. 5.1 The signal transduction pathways involved in the induction of CTGF through the FP receptor. FP cells were treated with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis under Experimental Procedures. In some cases, the cells were pretreated with either vehicle, 20 μM Bay43-9006 for 1 hour or 1 ng/ml Toxin B for 16 hours (**A, D**). In other cases, the cells were transfected with vehicle, Ras dominant negative construct (Ras DN) or TCF4 dominant negative construct (TCF4 DN) for 16 hours (**B, C**) before adding $PGF_{2\alpha}$. In the the upper panels, antibody against CTGF is used. The blots in the upper panels are re-probed with antibody against vincullin and the results are shown in the lower panels. Immunoblotting results are representative of three experiments with each antibody and condition.

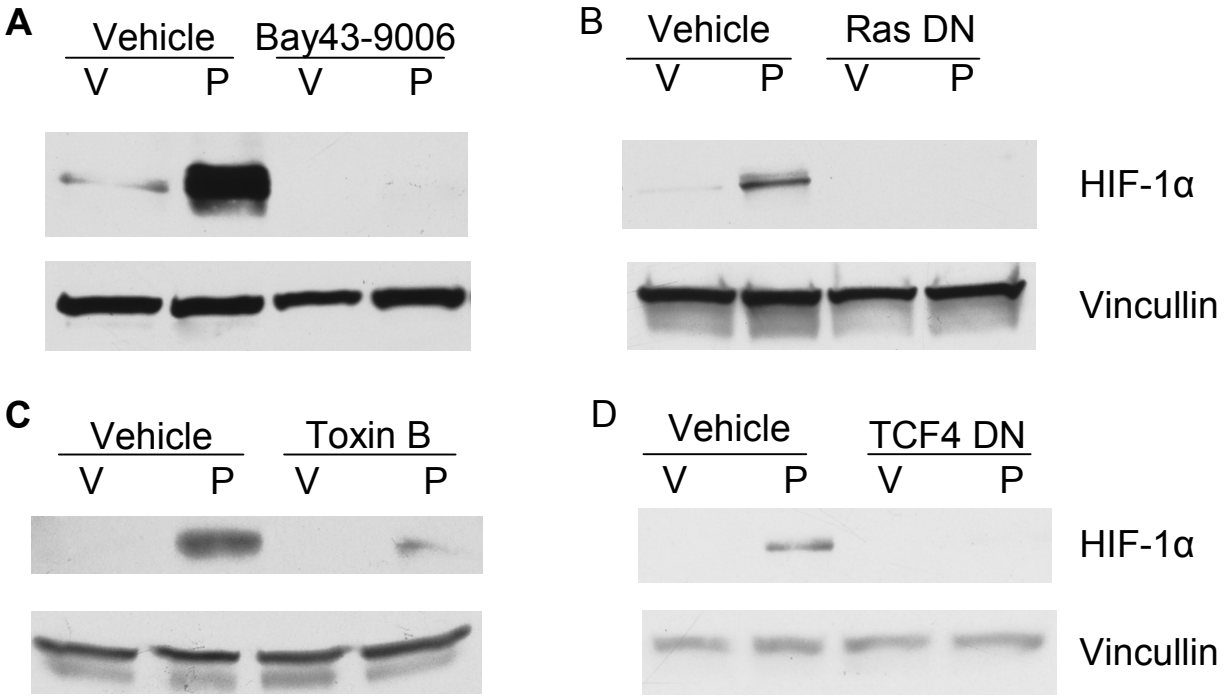

Fig 5.2 The signal transduction pathways involved in the induction of HIF-1α through the FP receptor. A, FP cells were treated with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures.** In some cases, the cells were pretreated with either vehicle, 20 μM Bay43-9006 for 1 hour or 1 ng/ml Toxin B for 16 hours (**A, D**). In other cases, the cells were transfected with vehicle, Ras dominant negative construct (Ras DN) or TCF dominant negative construct (TCF DN) for 16 hours (**B, C**) before adding $PGF_{2\alpha}$. In upper panels, antibody against HIF-1α is used. The blots in the upper panels are re-probed with antibody against vincullin and the results are shown in the lower panels. Immunoblotting results are representative of three experiments with each antibody and condition.

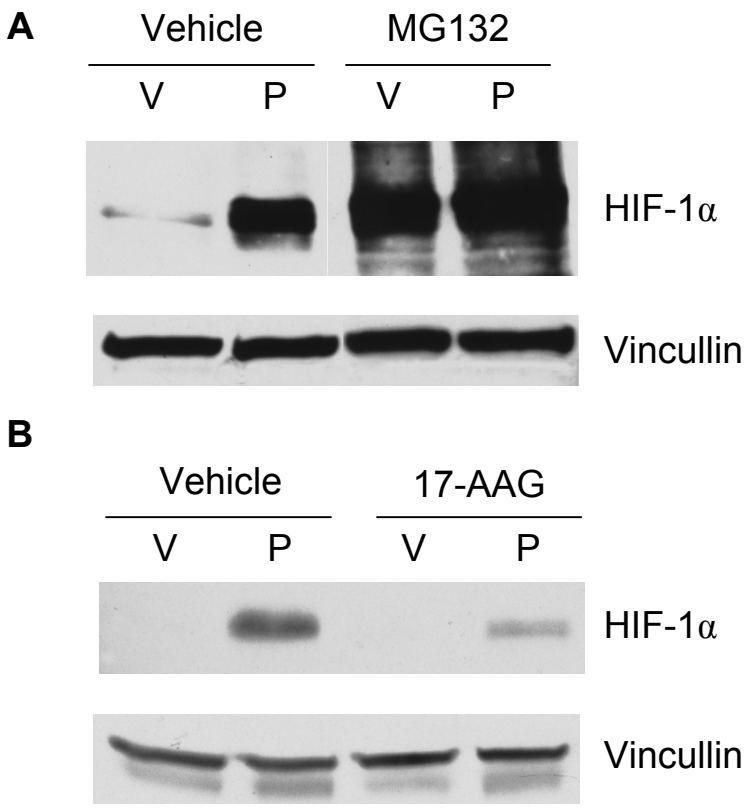

Fig 5.3 Protein degradation plays a major role in the gene regulation of HIF-1α through the FP receptor. FP cells were treated with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 6 h at 37 °C and were then immediately subjected to immunoblot analysis under **Experimental Procedures.** In some cases, the cells were pretreated with either vehicle, 50 μM MG-132 for 1 hour or 1 μM 17-AAG for 16 hours (**A, B**) . In upper panels, antibody against HIF-1α is used. The blots in the upper panels are re-probed with antibody against vincullin and the results are shown in the lower panels. Immunoblotting results are representative of three experiments with each antibody and condition.

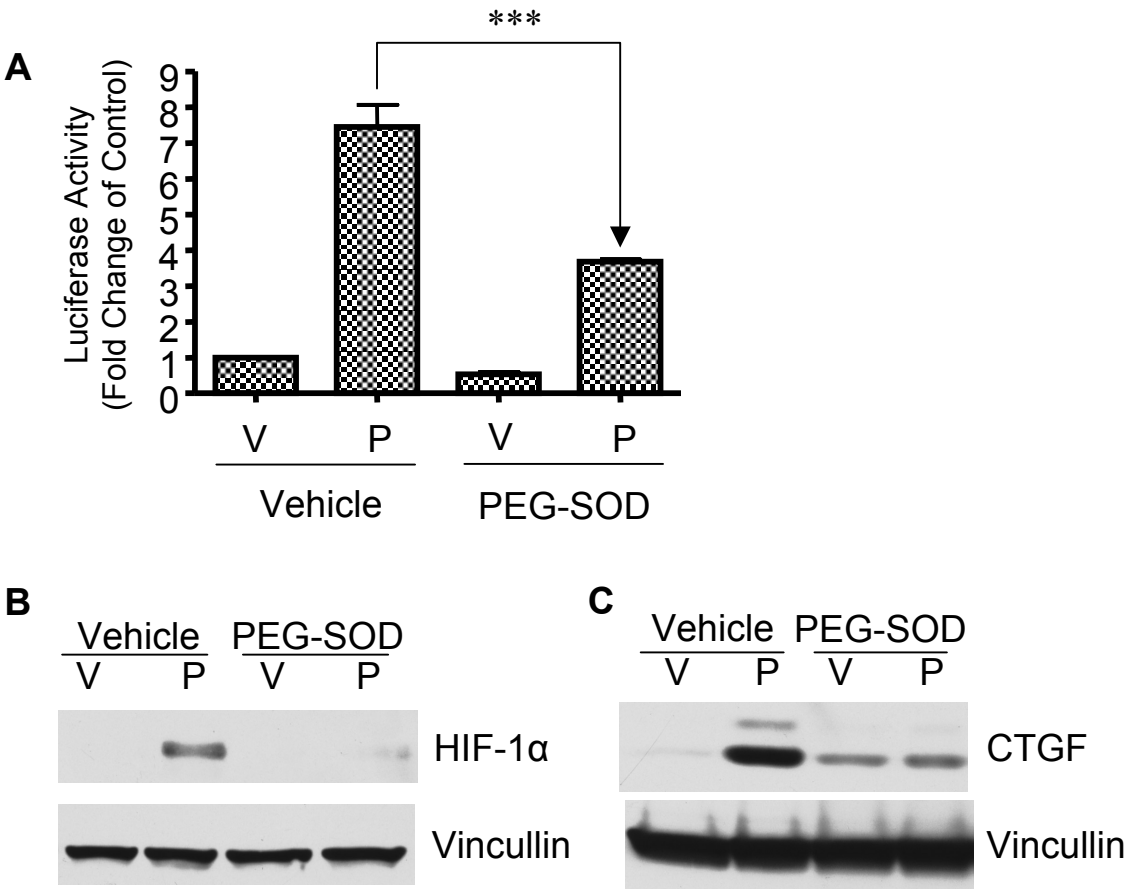

Fig 5.4 ROS is involved in the activation of TCF/β-catenin pathway and induction of HIF-1α through the FP receptor. A, FP cells were pretreated with either vehicle or 83 μg/ml PEG conjugated SOD (PEG-SOD) for 15 minutes followed by treatment with either vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 16 h. Shown is one representive experiment out of three independent experiments. Each point shown is the SEM of triplicate. Data are normalized to the vehicle-treated FP-expressing cells as 1. ***, $P<0.001$, as compared with vehicle-treated FP cells. **B**, FP cells were pretreated with 93 μg/ml PEG-SOD for 15 minutes followed by treatment with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 6 h at 37 °C. The cells lysates were subjected to immunoblot analysis under **Experimental Procedures.** In the upper panels, antibody against HIF-1α is used. The blots in the upper panels are re-probed with antibody against vincullin and the results are shown in the lower panels. **C**, as same as B, the only difference is that anti-CTGF antibody is used instead of HIF-1α. Immunoblotting results are representative of three experiments with each antibody and condition.

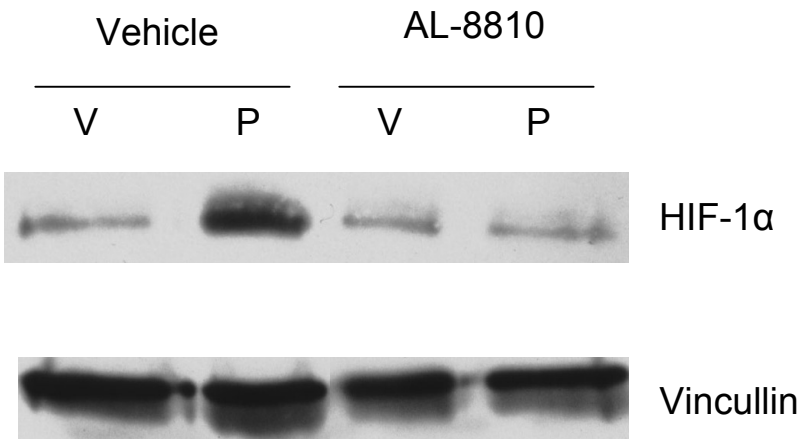

Fig. 5.5 Regulation of HIF-1α through the FP receptor in an endogenous system. Human microglia cells were pretreated with 10 μM AL-8810 for 15 minutes followed by treatment with vehicle (V) or 1 μM $PGF_{2\alpha}$ (P) for 3 h at 37 °C. The cells lysates were subjected to immunoblot analysis under **Experimental Procedures.** In upper panels, antibody against HIF-1α is used. The blots in the upper panels are re-probed with antibody against vincullin and the results are shown in the lower panels.

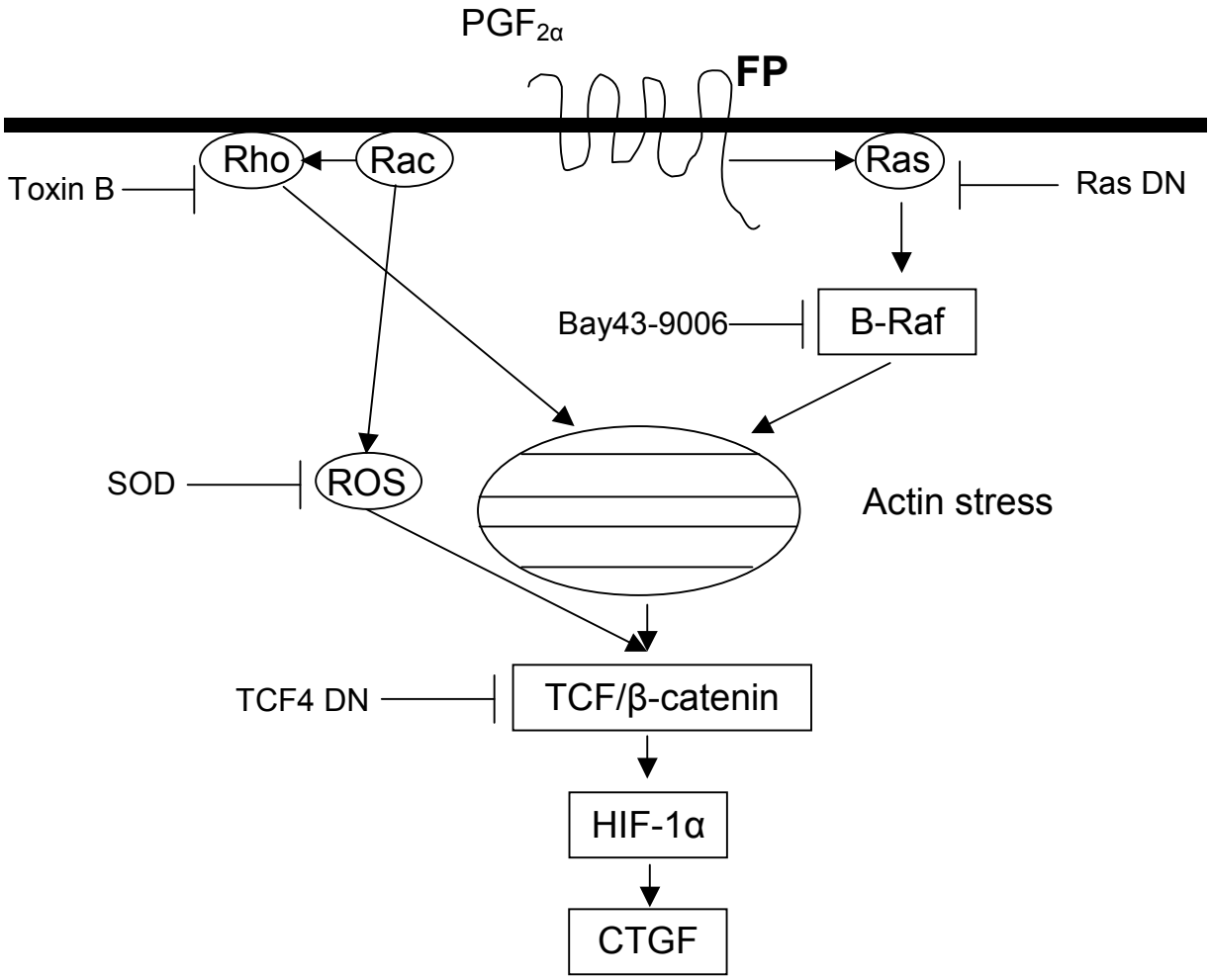

Fig 5.6 The signal transduction pathways involved in the induction of CTGF by the activation of the FP receptor. The FP receptor is capable of activating Ras, which in turn activates B-Raf. In the same time, Ras activates Rac, which activates Rho. Activated B-Raf converges with Rac/Rho pathway to induce the formation of actin stress fiber. In the same time, Rac activates NOX, which generates ROS. ROS converge with actin stress fiber to activate TCF/β-catenin pathway. The activation of TCF/β-catenin leads to the induction of HIF-1α and CTGF.

CHAPTER SIX

CONCLUSIONS AND FUTURE STUDIES

6.1 Conclusions

FP receptor, which is a member of the GPCR super family, has important biological functions. Because of its important biological functions, FP has been a drug target. To further exploit the FP as a drug target, it is necessary to elucidate the signal transduction pathways initiated from FP receptors. Some of these signal transduction pathways have been identified. But overall, the signal transduction pathways through the FP receptor have not been well characterized, especially the signal transduction pathways leading to gene regulation.

In the present study, we tried to identify some signal transduction pathways leading to the gene regulation through the FP receptors. In the beginning, we used cDNA microarray to identify the gene regulated by the agonist stimulated FP receptor. Then we confirmed the results from cDNA microarray analysis using Northern blot analysis and Western blot analysis. After the confirmation, three genes, EGR-1, Cyr61 and CTGF, were chosen for dissection of signal transduction pathways leading to their induction through the FP receptor. During this process, we found that Ras was activated through the FP receptor. The activation of Ras leads to three different downstream signal transduction pathways. The first is the activation of the C-Raf kinase. Activated C-Raf activates MAPK, which leads to the induction of EGR-1. The second is the activation of B-Raf. The third is the activation of Rac, which activates Rho. Rho converges with B-Raf to induce the actin stress fiber formation. The Rac also activates NOX, which leads to the generation of

ROS. The formation of actin stress fiber converging with ROS leads to the activation of TCF/β-catenin pathway. The activation of TCF/β-catenin induces the Cyr61 and HIF-1α, which up-regulates the expression of CTGF. All the signal transduction pathways through the FP receptor identified in this study are summarized in Fig. 6.1. Identification of these signal transduction pathways and crosstalks through the FP receptors, some of which are novel, may provide a better understanding of the role of FP in the diseases, specifically, cancer and heart disease.

The FP receptor has been found in multiple cancers, including lung cancer (Fang et al., 2004), endometrial adenocarcinoma (Sales et al., 2004) and skin cancer (Muller et al., 2000). However, the role of FP in cancer is not well-established. The only work concerning the roles of FP in cancer was carried out in endometrial adenocarcinoma (Jabbour et al., 2005). In the present study, we identified multiple induced proteins and signal transduction pathways leading to these inductions activated through the FP receptor. CTGF (Aikawa et al., 2006), Cyr61 (Menendez et al., 2003) and HIF-1α (Kimbro et al., 2006), which are identified to be induced through the FP receptor, all play important roles in cancer. The signal transduction pathways involved in the induction of these proteins, including TCF/β-catenin, Ras small G protein, Rho small G protein and B-Raf, is also well-known cancer-related signal transduction pathway. Moreover, the mechanisms revealed about the gene-regulation through the FP receptor provide some clues to how the FP receptor may be involved in cancer. For example, CTGF, Cyr61 and HIF-1α can be induced in hypoxia and are all involved in the angiogenesis of cancer

tissue (Chang et al., 2006; Kimbro et al., 2006; Menendez et al., 2003). Besides the roles of these proteins in cancer angiogenesis, these proteins may be involved in a positive feedback loop to amplify the signal transduction for cancer. Cyr61, a secreted protein whose receptor is an intergrin, can activate the TCF/β-catenin pathway (Menendez et al., 2003). Considering the activation of TCF/β-catenin pathway through the FP receptor (Fujino et al., 2001), the induction of Cyr61 through the FP receptor may further amplify the TCF/β-catenin pathway, a well-known pathway leading to the tumorigenesis. The other example is that HIF-1α can up-regulate the expression of COX-2 (Kaiti et al., 2006), which is responsible for the production of $PGF_{2\alpha}$. $PGF_{2\alpha}$ stimulates the FP receptor and induces HIF-1α, which may induce the expression of COX-2 and lead to more production of $PGF_{2\alpha}$. This positive feedback loop may be crucial for tumorigenesis. According to these results, FP may play important roles in cancer. Further studies are necessary to address this question, which will probably lead to a new cancer therapeutic target.

FP receptor has also been shown to be involved in the heart hypertrophy (Adams et al., 1996; Kunapuli et al., 1998). However, all these experiments were carried out in rats. We demonstrated the induction of EGR-1 and RAS/Raf kinase/MEK1/2 pathways leading to the induction of EGR-1 through human FP receptor. EGR-1 and all these signal transduction pathways have been involved in heart hypertrophy (Buitrago et al., 2005; Proud et al., 2004; Xiao et al., 2001). Moreover, ROS, probably superoxide ion, a well-known inducer of hypertrophy (Maccarthy et al., 2001), was found to be generated

through the human FP receptor. All these results suggest a mechanism for FP-dependent heart hypertrophy and the possibility of $PGF_{2\alpha}$ induced heart hypertrophy in humans.

Heart disease and cancer are the two leading causes of death in the US. Our study indicates the potential roles of the FP receptor in the causes of these two deadly diseases. Further study may lead to use of the FP as the therapeutic target. Actually, FP may be a very good therapeutic target because all the drugs targeting FP receptor have low toxicity. For example, FP receptor agonist $PGF_{2\alpha}$ has been widely used for inducing labor in clinic (Kelly et al., 2001). The other FP receptor agonist Bimatoprost has also been successfully used to treat glaucoma in clinic. Moreover, COX inhibitors, which can inhibit the production of $PGF_{2\alpha}$, have been widely used in clinic and proven to be safe in most cases. Based on these results, we speculate that manipulation of the FP receptor, probably using an antagonist, is most likely low-toxicity. More interestingly, COX inhibitors have been tried in clinic to prevent heart disease (Sawanyawisuth et al., 2005) and cancer (Bertagnolli et al., 2006). However, heart complications of rofecoxib (Vioxx) raise questions of the safety of COX, especially COX-2 inhibitors. We think that the problem is the broad targeting of COX-2 inhibitors. For example, PGI_2 can be inhibited by COX inhibitors and PGI_2 inhibition results in hypercalemia. PGE_2 inhibition results in sodium retention, which leads to hypertension, peripheral edema and potentially, exacerbation of heart failure (Sanghi et al., 2006). More specific inhibition of prostaglandin receptors, such as the FP antagonist, provides safer alternatives.

6.2 Future Studies

According to our study and previous literature (Adams et al., 1996; Kunapuli et al., 1998), we propose that EGR-1 is the potential mechanism of $PGF_{2\alpha}$-induced heart hypertrophy. In future study, we would like to test the hypothesis in mice because this model is more physiological relevant in heart hypertrophy.

First, we would like to test this hypothesis in FP knockout mice. FP knockout mice are healthy except the defect in parturition (Sugimoto et al., 1997). There is also a method called transverse aortic constriction (TAC) to induce the heart hypertrophy in mice and TAC-induced heart hypertrophy is decreased in EGR-1 knockout mice (Buitrago et al., 2005). We will compare the TAC-induced heart hypertrophy in FP knockout and wild type mice. The expected results are that TAC-induced heart hypertrophy is decreased in FP knockout mice compared to that in wild type.

Second, we would like to test this hypothesis in EGR-1 knockout mice. EGR-1 knockout mice are viable (Buitrago et al., 2005). A FP agonist fluprostenol has been successfully to induce the heart hypertrophy in rat (Lai et al., 1996). We will try to use the same drug to induce the heart hypertrophy in mice. Then, we will compare the fluprostenol-induced heart hypertrophy in EGR-1 knockout mice and wild type mice. The expected results are that fluprostenol-induced heart hypertrophy is decreased in EGR-1 knockout mice.

The above experiments will provide the evidence of the role of FP in heart hypertrophy. This may pave the way to the study of use FP as a drug target for treatment of heart hypertrophy.

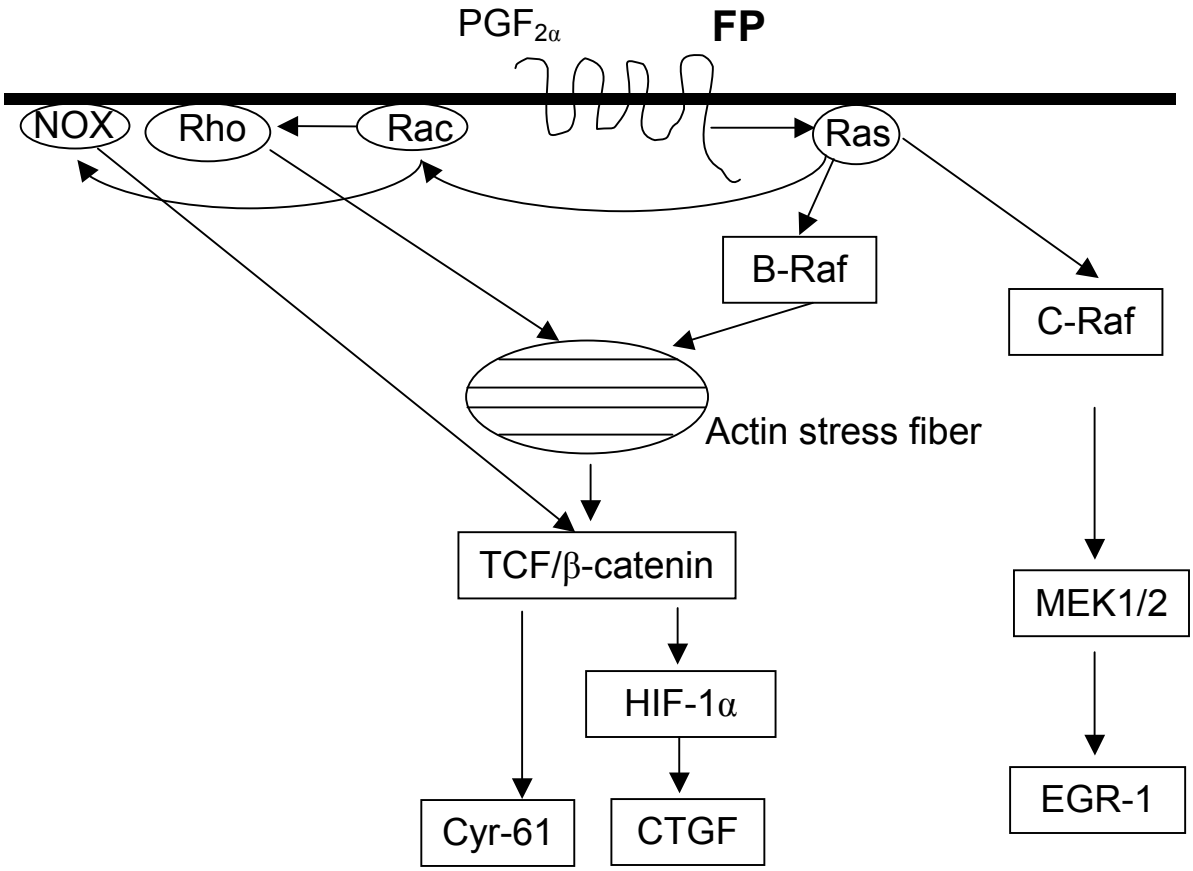

Fig 6.1 The novel signal transduction pathways by the activation of the FP receptor. The FP receptor is capable of activating Ras, which in turn leads to the activation of C-Raf and MEK1/2. The activation of MEK1/2 induces the EGR-1, which may be necessary for the $PGF_{2\alpha}$-dependent heart hypertrophy. Ras also activates B-Raf. Moreover, Ras can activate Rac, which activate Rho. Activated B-Raf converges with Rac/Rho pathway to induce the formation of actin stress fiber. In the same time, Rac activates NOX, which generates ROS. ROS converge with actin stress fiber to activate TCF/β-catenin pathway. The activation of TCF/β-catenin leads to the induction of Cyr61, HIF-1α and CTGF. The induction of these genes may cause cancer.

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