이학석사학위논문

CXCR4 이형이량체화의 기능 연구

Functional studies on CXCR4 heterodimerization

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양 창 수

Functional studies on CXCR4 heterodimerization

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지도교수 : 허 원 기

이 논문을 이학석사 학위논문으로 제출함

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양창수 의 이학석사 학위논문을 인준함

2016년 2월

위 원 장 _____(인)

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ABSTRACT

G-protein coupled receptors (GPCRs) transit extracellular ques to a series of internal signals. Many GPCRs with different function are in charge of diverse signaling pathways. C-X-C chemokine receptor type 4 (CXCR4) is involved in a class of GPCRs which mediates cell migration and development of immune cells. In tumor cells, extensive studies have identified its distinct roles in development and metastasis. There have been constraints on clinical researches with this indispensable CXCR4, because direct down-regulation of it was catastrophic to cell viability. To deal with CXCR4, cancer cell-specific regulation of CXCR4, not globally affecting normal tissue, is required. Recent findings elucidated that GPCRs are more often found as functional dimers or oligomers rather than monomers. In this study, BiFC and co-internalization analysis were utilized to sort out GPCRs interacting with CXCR4. Indirect regulation of cyclic AMP and calcium signaling by CXCR4 dimerization

demonstrated in this study suggests new possibilities and directions of CXCR4-target drug research.

Key words : CXCR4, Heterodimerization, Drug repositioning, Cancer

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BiFC	Bimolecular fluorescence complementation
VN	N-terminal fragment of VENUS protein
VC	C-terminal fragment of VENUS protein
CMV	Cytomegalovirus
GFP	Green fluorescence protein
YFP	Yellow fluorescence protein
GPCR	G protein coupled receptors
CXCR4	Chemokine receptor 4
IP3	Inositol-1, 4, 5-triphosphate
DAG	Di-acyl glycerol
PLC	Phospholipase C

PLD	Phospholipase D
PI3K	Phosphoinositide 3 kinase

1. INTRODUCTION

1.1. GPCRs

G protein coupled receptors (GPCRs) compose a class of one of the largest known family of membrane proteins in human and are key molecules of pharmaceutical industry as targets of numerous drug researches. GPCRs have seven-transmembrane domain and facilitate cellular responses to a variety of extracellular signals like photons, small-molecules, peptides, and proteins (Lagerstrom and Schioth, 2008). In the presence of such ligands, GPCRs have diverse signaling pathways ranging from smell, vision, and taste to cardiovascular, endocrine, neurological, and reproductive functions. Enhanced level of expression in numerous cancer cells suggested some distinct roles of GPCRs in cancer cell development and metastasis (Scott *et al*., 2003; Smith *et al*., 2004; Walser and Fulton, 2004). These various functions of GPCRs make them one of the main targets for drug research (Overington *et al*., 2006; Tyndall and Sandilya, 2005). A recent report showed GPCRs are the primary

targets of current drugs out in the market (Overington *et al*., 2006). Recent studies have discovered that many of functional GPCRs exist as the heterodimeric form, not monomer (Angers *et et al*., 2002; Filizola and Weinstein, 2002; Yi *et al*., 2001). Therefore, it is more important to investigate the roles of GPCRs as heterodimers according to the expression pattern than to focus only on monomers (Mellado *et al*., 2002; Satake *et al*., 2013).

1.2. CXCR4

Chemokine receptors regulate the cell growth and migration in various cell types including leukocytes (Baggiolini, 1998; Moser *et al*., 2004). It is a major mediator of immune responses like inflammation and allergic hypersensitivity. (Baggiolini and Dahinden, 1994). Chemokine-induced chemotaxis recruit immune cells to the inflamed area. Interleukin-mediated immune activation is also specifically induced by chemokine (Baggiolini *et al*., 1994). It is also noticeable that in lymphocytes with enhanced level of chemokine receptor, human immunodeficiency virus infection is promoted (Bleul *et al*., 1997; Moore and Koup,

1996).

Chemokine (C-X-C motif) receptor 4 (CXCR4) is a GPCR, and stromal cell-derived factor-1 (also called SDF-1 and CXCL12) is the specific agonist for CXCR4 (Murphy *et al.*, 2000). The expression of CXCR4 is very low or absent in most of normal tissues while it is enhanced in many types of cancer tissues (Sun *et al.*, 2010). Depending on cell types, the function of CXCR4 is diverse: regulation of leukocyte stem cell mobilization and homing in normal tissues (Caruz *et al.*, 1998). CXCR4 is also responsible for cancer progression, metastasis and human immunodeficiency virus infection (Sun *et al.*, 2010). Elevated expression of CXCR4 has direct effect on survival and proliferation of tumor cells. Malignancy and metastasis formation in several tumor types are also related to its expression pattern (Fulton, 2009; Walser and Fulton, 2004; Zlotnik, 2006).

1.3. GPCR signaling

Upon activation of GPCR, diverse signaling pathways are

activated, leading to a number of responses (Busillo and Benovic, 2007). GPCR is coupled with G-protein hetero-trimer, which consists of G_{α} $G_{\beta\gamma}$ subunits and transduces the signal from GPCR at the arrival of a ligand (Rondard *et al.*, 2001). Variety of second messengers including inositol-1, 4,5-triphosphate (IP_3), diacyl glycerol (DAG) and arachidonic acid (for phospholipases), Ca^{2+} (for ion channels), cyclic AMP (for adenylyl cyclases) are at the downstream of G-protein mediated signal pathway (Mahajan and Tuteja, 2005). G_{α} regulates calcium channels, potassium channels, adenylyl cyclase, phospholipase C (PLC), phospholipase D (PLD) and protein kinases (Myers *et al.*, 1995; Perez and Karnik, 2005). G_{α} either stimulates or inhibits the activity of adenylyl cyclase, mediating the conversion of ATP to cyclic AMP (Birnbaumer, 2007). Stimulatory and inhibitory G_{α} are classified as $G_{\alpha s}$ and $G_{\alpha i/o}$, respectively. $G_{\alpha q}$, another class of alpha subunit, activates PLC that hydrolyzes Phosphatidylinositol 4,5-bisphosphate to DAG and IP_3 (Seifert *et al.*, 2003). CXCR4 signaling is blocked by Pertussis toxin, a $G_{\alpha i}$ specific blocker (Fields and Casey, 1997). It implies that CXCR4 acts with heterotrimeric G-protein

composed with $G_{\alpha i}$ subunit (Figure 1).

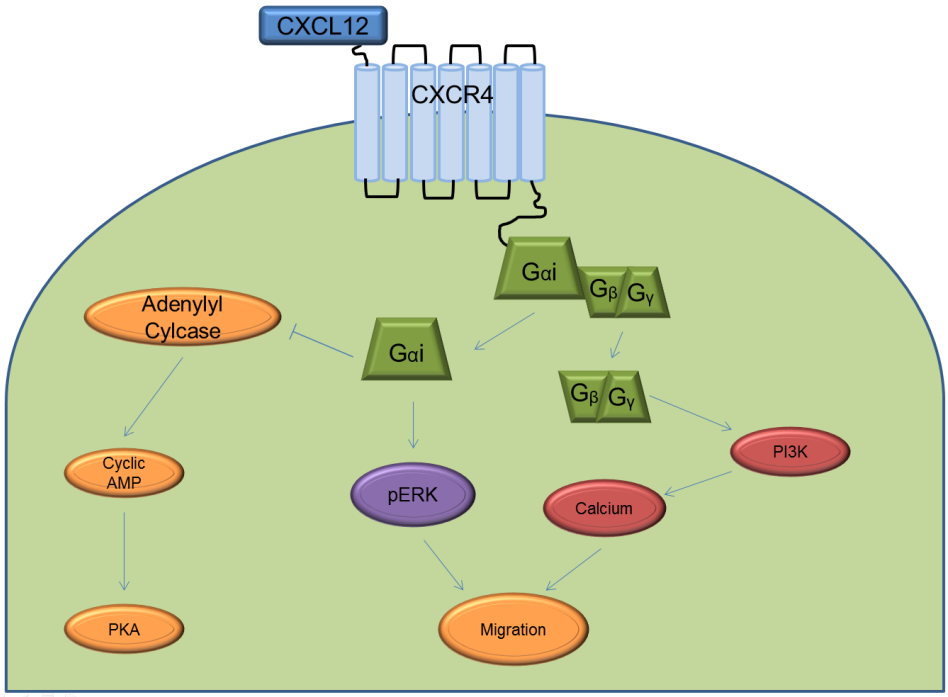


Figure 1. A schematic of the CXCR4-CXCL12 intracellular signaling pathways

CXCR4 acts with heterotrimeric G-protein composed with $G_{\alpha i}$ subunit. $G_{\alpha i}$ regulates protein kinase A (PKA) activity through inhibition of adenylyl cyclase. $G_{\beta\gamma}$ subunit transduces the signal via PI3K, which induce calcium mobilization in the cells. Calcium mobilization is related to migration.

1.4. GPCR heterodimerizaiton

An emerging issue in GPCR signaling is heterodimerization (Angers *et al*., 2002). Currently, various GPCRs have been verified to function as heterodimer (Angers *et al*., 2002; Levoye *et al*., 2006; Satake *et al*., 2013). At the dawn of GPCR studies, the interaction with heterotrimeric G protein was magnified as the important issue. Several pharmacological follow-up studies discovered diverse functions of GPCRs as dimer or oligomer (Bouvier, 2001). Not only homodimers but heterodimers and hetero-oligomers were found (Limbird *et al*., 1975; Potter *et al*., 1991; Wreggett and Wells, 1995), Biochemical approaches also supported the heterodimerization of GPCRs (Herberg *et al*., 1984; Peterson *et al*., 1986; Venter *et al*., 1984). It has been proved that GPCR heterodimerization is crucial for ligand binding affinity and signaling (Figure 2) (Levoye *et al*., 2006; Satake and Sakai, 2008). Extensive studies have followed to support the heterodimerization of GPCR.

Clinical approaches adopting the concept of heterodimeric

GPCR was applied. For example, the heterodimer of 5-hydroxytryptamine (serotonin) receptor 2A and metabotropic glutamate receptor 2 can alter the activity of its interaction partner. Specific inverse agonist of 5-hydroxytryptamine receptor 2A can activate metabotropic glutamate receptor 2 like it's agonist (Ellaithy *et al.*, 2015). Along with previous research, the importance of GPCR heterodimerization is gathering interest. Novel functions of GPCRs in their heterodimeric states remain elusive. It is assumed that the function of GPCR heterodimers varies according to the expression pattern of the protein in cells. Therefore GPCR heterodimer study is essential to elucidate GPCR's exact function *in vivo*.

Extensive exploration on GPCR heterodimerization revealed its more detailed functions along with pre-estimated roles as monomer. Heterodimerization studies up to now, however, have not clearly elucidated which GPCRs are involved in heterodimerization and what are the major physiological functions of heterodimers in normal tissues.

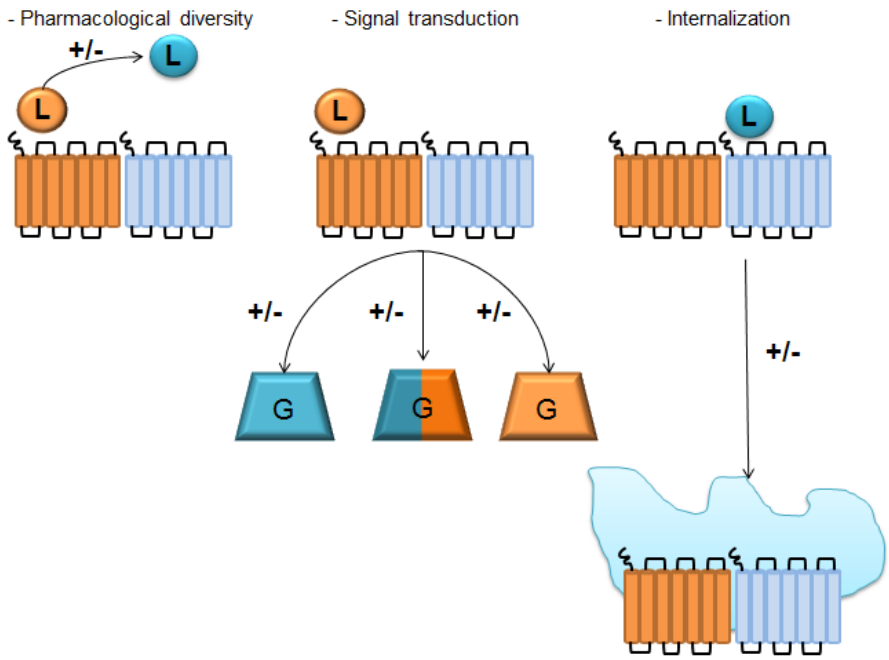


Figure 2. Schematic diagram presenting the roles of GPCR heterodimerization

GPCR heterodimerization regulates ligand binding affinity and signaling pattern. Some GPCRs are co-internalized by interactor.

1.5. Bimolecular fluorescence complementation (BiFC) assay

Bimolecular fluorescence complementation (BiFC) assay is a powerful method used to detect protein-protein interactions. Not only can it show the interaction of proteins, BiFC assay also provides information about the sites of interaction and diverse changes of location in accordance with conditions in living cells. Venus protein, one of yellow fluorescent protein variants, can be applied in BiFC assay (Nagai *et al.*, 2002). In the BiFC assay, YFP protein is halved, and these non-fluorescence fragments VN and VC are fused to proteins. It is recovered as they approach each other when interaction occurs between proteins each fragment is attached to. It enables the validation of the interaction by visualizing *in vivo* images (Hu *et al.*, 2006; Kerppola, 2006).

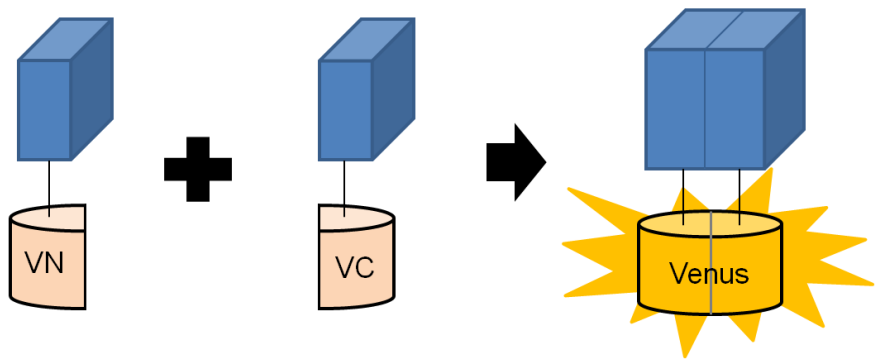


Figure 3. The principle of the BiFC assay

YFP protein is halved, and these non-fluorescence fragments VN and VC are fused to proteins. Fluorescence recovered as they get closed each other when interaction occurs between proteins each fragment.

2. MATERIALS AND METHODS

2.1. Cell Lines

HEK293A and MDA-MB-231 cells were purchased from the American Type Culture Collection.

2.2. Cell Culture

HEK293A and MDA-MB-231 cells were grown in Dulbelcco's modified Eagle's medium (DMEM; HyClone Laboratories) supplemented with glucose, 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories), 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA), and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO_2 .

2.3. Transfection

HEK293A Cells were transiently transfected by Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol.

2.4. Infection

Adenoviral infection was performed at multiplicity of infection = 20 for MDA-MB-231 cells. Cells were incubated in culture media with the mixture of viruses. At 4 hr post-infection, media were added into the mixture. Cells with over 90% of transduction efficiency were subjected to calcium assay.

2.5. Bimolecular Fluorescence Complementation (BiFC) Assay

HEK293A cells were cultured on a black wall 96-well clear-bottom plate at 37℃ in a 5% CO_2 for 24 h. The cells were transiently co-transfected with pcDNA3.1-CXCR4-VN and pcDNA3.1-GPCR-VC. 24 h after transfection HEK293A cells were washed twice in DPBS (HyClone). The cells were fixed with DPBS containing 2% paraformaldehyde at room temperature for 30 min, followed by an additional two washes with DPBS and then stained by DPBS containing 1 mg/ml of Hoechst 33342 (Thermo Fisher Scientific) for 30 min. The BiFC assay was performed on a Nicon Eclipse E1 microscope.

2.6. Co-internalization Assay

HEK293A cells were cultured on a black wall 96-well clear-bottom plate at 37℃ in a 5% CO_2 for 24 h. The cells were transiently co-transfected with pcDNA3.1-CXCR4-GFP and pcDNA3.1-GPCR-VC. 24 h after transfection, HEK293A cells were washed twice in DPBS. The cells were fixed with DPBS containing 2% paraformaldehyde at room temperature for 30 min, followed by an additional two washes with DPBS and then stained by DPBS containing 1 μg/ml of Hoechst 33342 (Thermo Fisher Scientific) for 30 min. The Co-internalization images were acquired with IN cell Analyzer 2000 and analyzed using Investigator software (GE Healthcare).

2.7. Cyclic AMP Assay

The concentration of cyclic AMP within cells was determined by ENVISION (PerkinElmer) using cAMP parameter assay kit (R&D Systems Europe Ltd) according to the manufacturer's

instructions.

2.8. Calcium Assay

Before 24 hr, MDA-MB-231 cells were seeded in 96-well plate (Corning) at 2x10^4 cells per well and then cells were transiently infected by adeno viral vector system. At 24 hr post-infection, calcium concentration of cells was determined using FLIPR Calcium 6 Assay Kit and FlexstationIII (Molecular Devices) according to the manufacturer's instructions.

3. RESULTS

3.1. Validation of GPCRs expression and function

Ahead of their heterodimerization, the expression and localization of GFP-fused GPCR constructs was confirmed. Constructs under the control of cytomegalovirus (CMV) promoter were transfected to the HEK293A cells. GFP Signals of all the GPCRs utilized in the study were found in the membrane normally (Figure 4a). Cyclic AMP level which was elevated by forskolin, the adenylyl cyclase activator, was suppressed by CXCL12 in CXCR4 over-expressed cells (Figure 4c), and it was antagonized by AMD3100, the CXCR4 antagonist (Figure 4c). Another signaling pathway, calcium mobilization was also induced by the CXCR4-CXCL12 axis (Figure 4b). Based on these two experiments, the expression and localization of functional CXCR4 was confirmed.

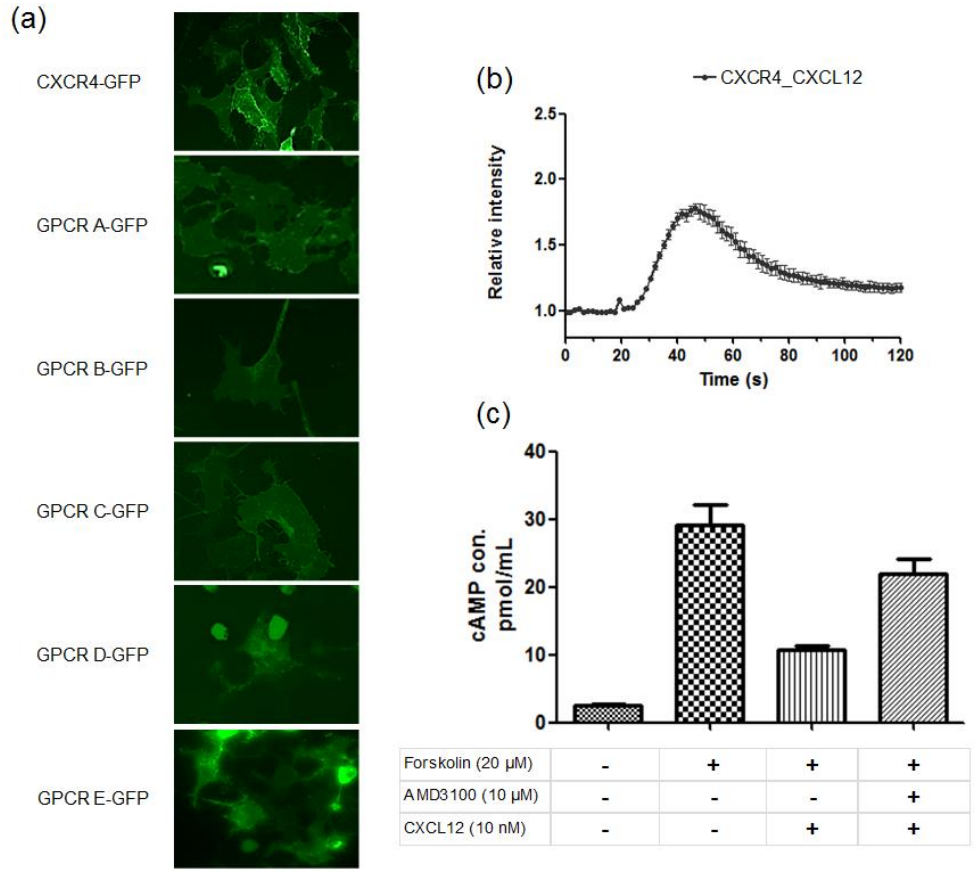

(a)
CXCR4-GFP
GPCR A-GFP
GPCR B-GFP
GPCR C-GFP
GPCR D-GFP
GPCR E-GFP
(b)
CXCR4_CXCL12
Relative intensity
2.5
2.0
1.5
1.0
0
20
40
60
80
100
120
Time (s)
(c)
cAMP con. pmol/mL
40
30
20
10
0
Forskolin (20 μM) - + + +
AMD3100 (10 μM) - - - +
CXCL12 (10 nM) - - + +

Figure 4. GPCRs expression and function

(a) GFP fused GPCRs were detected under the microscope. CXCL12-induced calcium reaction was observed in CXCR4 over-expression cell lines. Cells were stained with calcium 6 dye and analyzed by Flexstation III. (b) CXCL12 was added at 20 s, followed by dramatic increase in cytosolic calcium. (c) The normal state of G_i coupled signaling of CXCR4 was detected by the cyclic AMP assay, and the function of CXCR4 was antagonized by AMD3100, one of its well-known antagonists.

3.2. CXCR4 interacts with several GPCRs.

To investigate interactions between CXCR4 and GPCRs, BiFC assay was utilized. CXCR4 and GPCRs were tagged with VN and VC, respectively. A series of GPCRs (GPCR A, GPCR B, GPCR C, GPCR D and GPCR E) once at a time and CXCR4 were co-expressed by transfection and examined using fluorescence microscope. YFP fluorescence was recovered in GPCR-A, B, C, and D, suggesting that their VN is in close proximity with the VC-tagged CXCR4 but not GPCR E (Figure 4e). However, the BiFC data cannot present exact evidence of functional interaction with CXCR4.

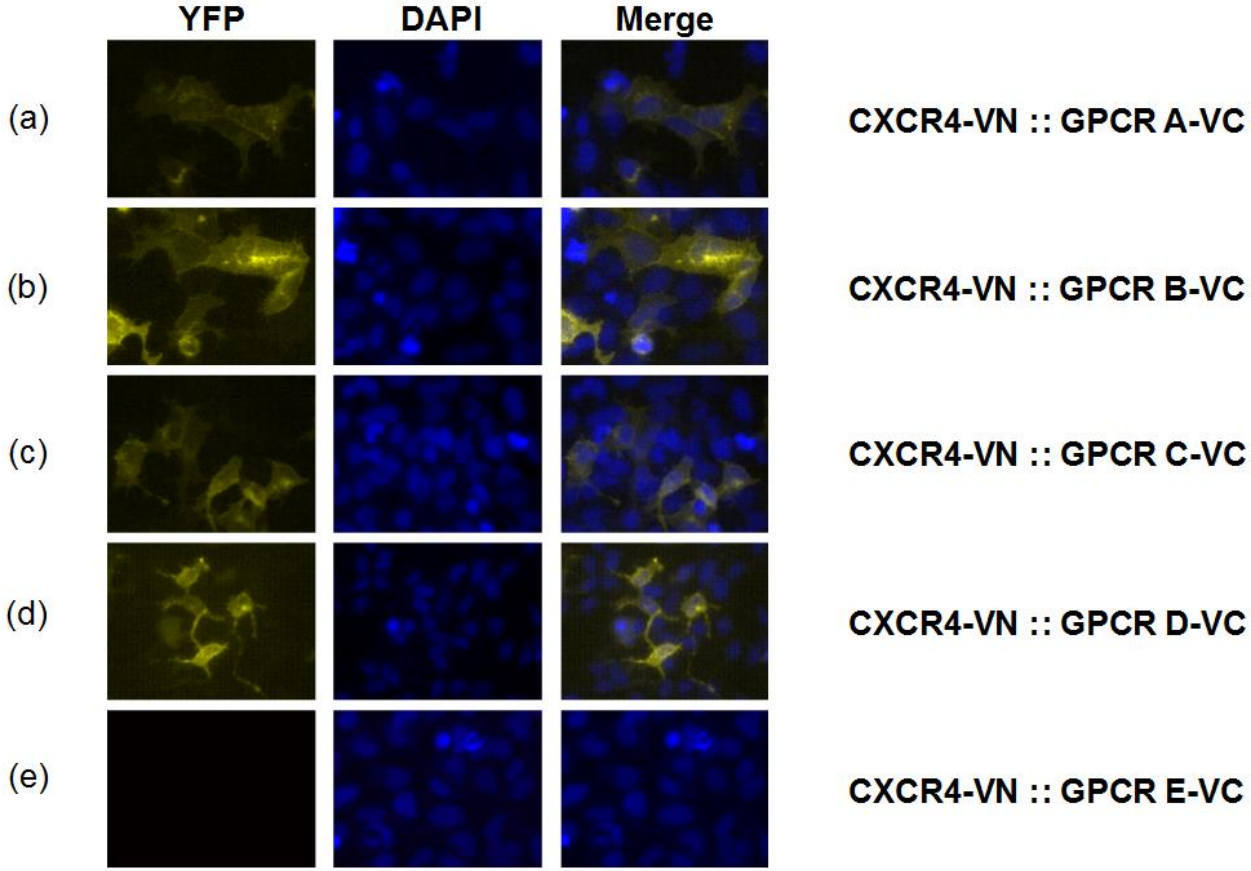

YFP
DAPI
Merge
(a)
CXCR4-VN :: GPCR A-VC
(b)
CXCR4-VN :: GPCR B-VC
(c)
CXCR4-VN :: GPCR C-VC
(d)
CXCR4-VN :: GPCR D-VC
(e)
CXCR4-VN :: GPCR E-VC

Figure 5. BiFC assay of CXCR4 with GPCRs

BiFC assay was performed on CXCR4 and 5 GPCRs (GPCR A, GPCR B, GPCR C, GPCR D, and GPCR E). HEK293A cells were transiently transfected with pcDNA vector containing CXCR4 and GPCRs which were tagged VN and VC, respectively. Under the microscope, GPCR A, GPCR B, GPCR C, and GPCR E which were co-expressed with CXCR4 showed the YFP signals (a, b, c, and d), but not GPCR E (d). The location of interaction was expected on cell membrane where the GPCRs have function.

3.3. CXCR4 and GPCRs are located within enough to interaction.

In order to confirm the interactions between CXCR4 and GPCRs, co-internalization assay, another technique which can elucidate protein-protein interaction, was utilized. One of the interesting features in GPCRs is internalization for desensitization (Bohm *et al*., 1997). If two GPCRs are close enough to interact, it would be possible to detect the co-internalization of the two parts due to the ligand of the interaction partner. HEK293A cells transiently over-expressed CXCR4 and GPCRs which were tagged with GFP and VC were used for co-internalization assay. After 24h of transfection, I followed the localization of CXCR4-GFP before and after treatment of CXCL12. CXCR4 was internalized and had forming spot signals under treatment of CXCL12 (Figure 5). CXCR4, which was co-expressed with GPCR A, GPCR B, and GPCR D, was internalized by agonist A and B. Agonist A and B are respective agonists of GPCR A and B. It wasn't internalized when co-expressed with GPCR C (data not shown) and GPCR E. The data clearly shows that CXCR4 is in close proximity with

GPCR A, GPCR B, and GPCR D to interact with each other.

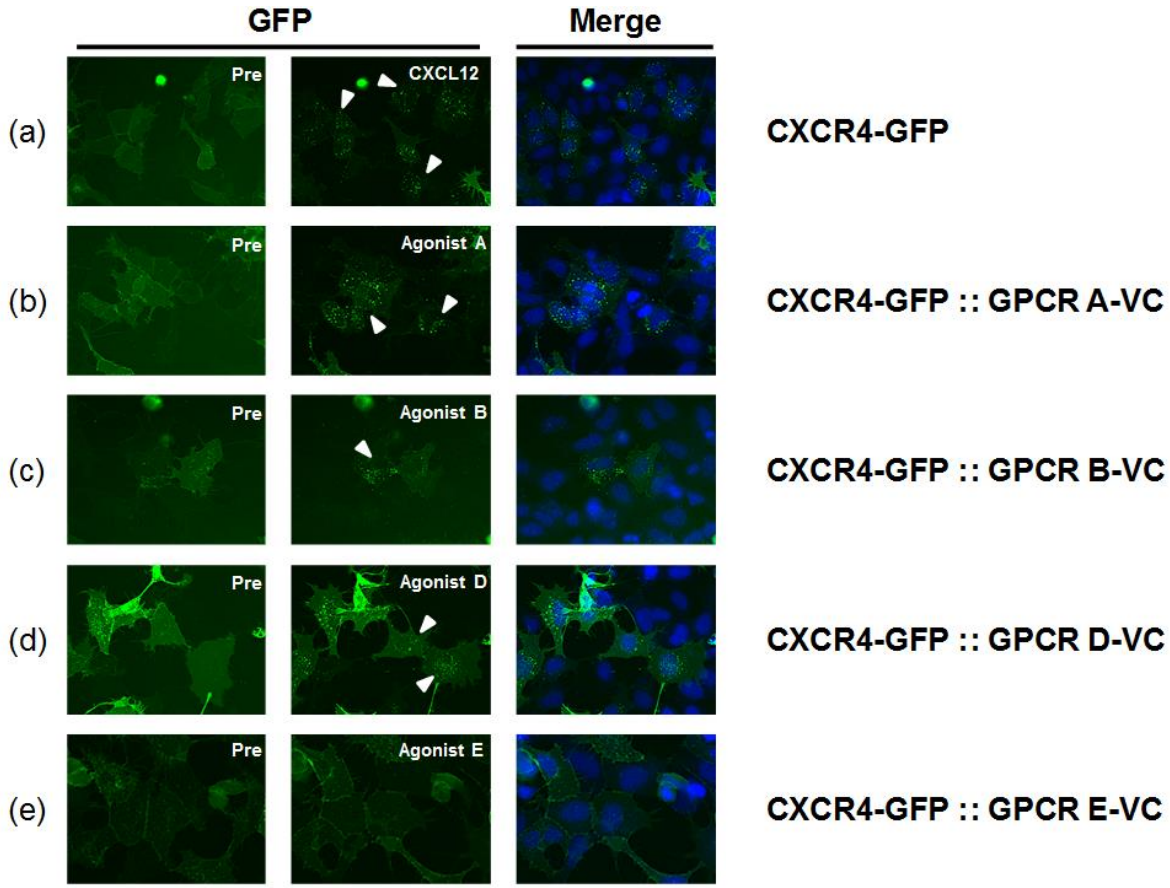

GFP
Merge
(a)
Pre
CXCL12
CXCR4-GFP
(b)
Pre
Agonist A
CXCR4-GFP :: GPCR A-VC
(c)
Pre
Agonist B
CXCR4-GFP :: GPCR B-VC
(d)
Pre
Agonist D
CXCR4-GFP :: GPCR D-VC
(e)
Pre
Agonist E
CXCR4-GFP :: GPCR E-VC

Figure 6. Co-internalization of CXCR4

GFP-tagged CXCR4 and VC-tagged GPCRs were transiently over-expressed in HEK293A cells. The procedure was undertaken on a 96 well plate to observe using INCELL analyzer. GFP was observed before and after CXCL12, agonist A, and agonist B addition to detect local changes of CXCR4 due to the molecules. Localization of CXCR4 before and 30 minutes after CXCL12 treatment was tracked. Hoechst staining was carried out with identical procedure. All images were obtained through INCELL. (a) CXCR4's internalization was confirmed through CXCL12, and co-internalized with GPCR A, GPCR B, and GPCR D (b, c, and d).

3.4. Heterodimerization with GPCRs alters the calcium influx and regulates cytosolic cyclic AMP level reduced by CXCR4-CXCL12 axis.

Data up to this point only shows physical interactions between CXCR4 and GPCRs. In order to find out if these physical interactions influence the signaling between the two interacting GPCRs, the effects of heterodimerization on calcium and cyclic AMP signaling pathways, the two essential signaling of CXCR4, were analyzed. CXCR4 and CXCL12 axis increase the cellular Ca^{2+} level and inhibit adenylyl cyclase A (Stantchev and Broder, 2001). At first, we examined the cytosolic calcium level of CXCR4. GPCRs are co-expressed in MDA-MB-231 cells using adenoviral vector system, and they were stained by Calcium 6 dye. Intracellular calcium level was analyzed using FlexstationIII. I found that the calcium influx induced by CXCL12 treatment in CXCR4-expressing cells was increased by GPCR A (Figure 6a), suppressed by GPCR B (Figure 6b), prolonged by GPCR C (Figure 6c), but not changed by a negative control GPCR D (Figure 6d).

Next, I investigated whether the cytosolic cyclic AMP level can be affeced by heterodimerization. CXCR4-CXCL12 axis transduces signals mediated with $G_{\alpha i}$ subunit. Inhibition of adenylyl cyclase A by $G_{\alpha i}$ subunit leads to decrease of cytosolic cyclic AMP level (Busillo and Benovic, 2007). In normal state, cytosolic cyclic AMP level is too low to distinguish CXCR4 activity. In this study, the level of Cytosolic cyclic AMP was elevated by treatment of forskolin, which is an activator of adenylyl cyclase (Rodriguez *et al*., 2013; Schneyer *et al*., 1983; Seamon and Daly, 1981). CXCR4 and GPCRs were transiently expressed in HEK293A cells. Cells were incubated with forskolin and CXCL12 for 15 min, and then cell lysates were prepared by boiling for 10 min. In the presence of forskolin, cytosolic cyclic AMP level was increased and decreased by CXCL12 (Figure 7a). In contrast with calcium assay, Only GPCR B affected on cyclic AMP signaling pathway of CXCR4 (Figure 7b). Interestingly, as the result of calcium assay, GPCR B suppressed the function of CXCR4 related regulation of cyclic AMP.

There have been numerous reports that CXCR4's expression level increases once a normal tissue becomes a cancer tissue (Kukreja *et al*., 2005; Salvucci *et al*., 2006), and the effects of CXCR4 on cancer development has been well researched (Beider *et al*., 2009; Du *et al*., 2014; Duda *et al*., 2011). But since CXCR4's functional activity level can also affect cancerization (Miller *et al*., 2008), this can be a new approach to studying the cancerization process. Accordingly, it suggests that the interaction between CXCR4 and GPCRs can influence CXCR4 signaling itself by simply forming a heterodimer with it.

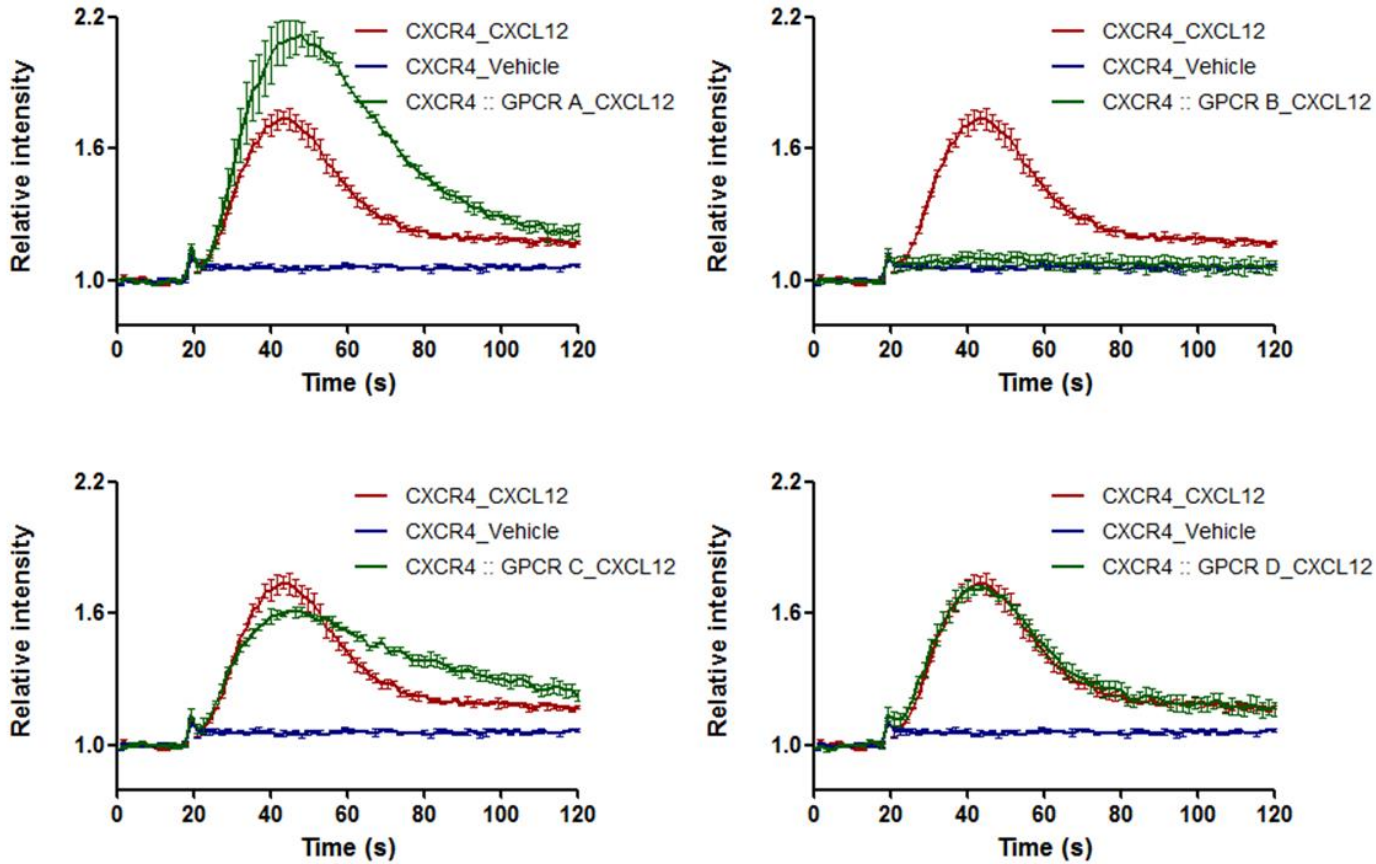
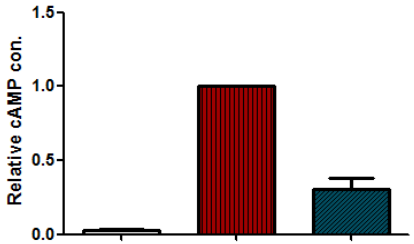

Relative intensity
2.2
1.6
1.0
0
20
40
60
80
100
120
Time (s)
CXCR4_CXCL12
CXCR4_Vehicle
CXCR4 :: GPCR A_CXCL12
CXCR4 :: GPCR B_CXCL12
CXCR4 :: GPCR C_CXCL12
CXCR4 :: GPCR D_CXCL12

Figure 7. Alteration of CXCR4-mediated calcium signaling by heterodimerization

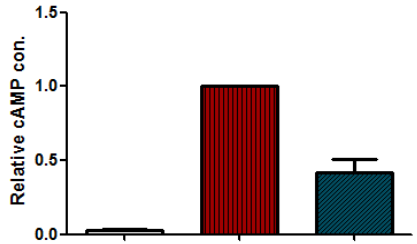
CXCR4 and GPCR A, B, C, D, and E were transiently expressed through Adenoviral vector system. 2 hr staining was done with Calcium 6 dye 24 hours after infection, and influx of calcium change was tracked through Flexstation III. CXCL12 was added at 20 seconds and its changes were measured for 2 minutes. There was no change in calcium intensity level when Vehicle was treated (Blue line), and this was compared to the calcium intensity level when calcium response occurs for cells in which CXCR4 is expressed and when the protein is forming a heterodimer. Heterodimer with GPCR A led to an increase in calcium signaling than when CXCR4 is alone (a), and the heterodimer with GPCR B led to a decrease (b). When the heterodimer was with GPCR C, the calcium level's increase pattern showed some difference (c). All data has been repeated 3 times, and relative intensity was obtained by taking the average value when nothing had been treated (1 sec ~ 20 sec).

(a)



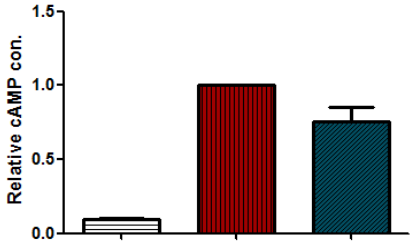
Forskolin (20 μM)	-	+	+
CXCL12 (10 nM)	-	-	+

(b)



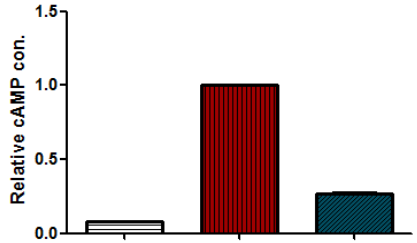
Forskolin (20 μM)	-	+	+
CXCL12 (10 nM)	-	-	+

(c)



Forskolin (20 μM)	-	+	+
CXCL12 (10 nM)	-	-	+

(d)



Forskolin (20 μM)	-	+	+
CXCL12 (10 nM)	-	-	+

Figure 8. Suppression of CXCR4-mediated cyclic AMP signaling by heterodimerization with GPCR B

CXCR4 and GPCRs were over-expressed on HEK293A cells. Before the measurement of cyclic AMP level, forskolin and CXCL12 were treated 20 μM and 10 nM respectively for 15 min. The increased cyclic AMP concentration when treated with Forskolin decreased when CXCR4 was over-expressed and CXCL12 was treated (a). There is no significant difference in CXCR4's function where GPCR A and GPCR C is co-expressed with CXCR4 (b and d), but the function of CXCR4 is supressed when co-expressed with GPCR B (c).

4. DISCUSSION

Malignant cells from at least 23 different types of cancer express the chemokine receptor CXCR4 and respond to its ligand CXCL12 (Balkwill, 2004). In addition, because CXCR4 has prominent influences on development of cancer and its metastasis, CXCR4's inhibition has been the target of various cancer therapies (Chen *et al.*, 2003; Duda *et al.*, 2011; Salvucci *et al.*, 2006; Singh *et al.*, 2010). Despite so, CXCR4's functions in normal tissues are of paramount importance so its direct inhibition causes a number of side effects (Hendrix *et al.*, 2004), a limit to appliance in medical clinics. Thus, if CXCR4 is controlled in a way its functions in normal tissues aren't affected, this could serve as a new direction for drug research which targets CXCR4. Previous research has mostly studied monomeric GPCR (Busillo and Benovic, 2007; Cherezov *et al.*, 2007; Gilman, 1987; Loza *et al.*, 2006; Singh and Chauthe, 2011). But since a variety of GPCRs interact with each other, it is essential to study GPCR interactome to discover its exact function in cells. Studies on

predicting GPCR pairs which form heterodimers and the function alteration which follows are yet meager in number (Honoo, 2013). If GPCR's interactome can be constructed through BiFC, a powerful tool which can find protein interaction, studies about GPCR's function will get an improvement in accuracy.

We were able to validate the possibility of indirectly controlling CXCR4's functions through this study. CXCR4 and multitude of GPCRs' interaction was confirmed through BiFC assay and Co-internalization assay. Some GPCR interactions shown by BiFC couldn't be found in the Co-internalization assay. A ligand binding to GPCR and initiating a signal transduction can cause a change in GPCR's structure. Thus, this structural shift can affect the protein's interaction; ligand binding can induce or inhibit heterodimerization (Hebert *et al*., 1996; Cvejic and Devi, 1997; Angers *et al*., 2000). Because of the possibility of consequent changes, future research on heterodimers when ligands are present is important, and we predict more precise studies on reported GPCR's characteristics will be possible. Only CXCR4's signaling was examined in this study, looking into the

effects of CXCR4 on cancer cells is left for future research. There have been plenty of research that report CXCR4's calcium signaling pathway, one of its many, is related to the development of cancer and metastasis, and it has also been published that repressing Ca^{2+} pathway also inhibits metastasis due to CXCR4 (Matthew *et al.*, 2004; Klein *et al.*, 2001; Soriano *et al.*, 2003). Therefore, the contents of this research about calcium signaling pathway control are likely to become an important proof of metastasis caused by CXCR4. If we can use tools such as migration assay and invasion assay to check various cell types' metastasis and its influence in their physiological roles, track the expression pattern changes of GPCRs specific to cancer patient's tissue that were found to share functional interaction with CXCR4 in this study, and verify whether the CXCR4's interactors are expressed in the patient's cancer cells, we can suggest indirect manipulation of CXCR4 as a new goal for cancer therapy research.

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국 문 초 록

G-protein coupled receptor (GPCR)은 세포 내에서 다양한 외부 환경의 신호를 일련의 정보로 전환한다. GPCR은 신호의 종류나 기능에 따라 다양하게 분류가 되는데, 이 중 C-X-C chemokine receptor type 4 (CXCR4) 는 정상 조직에서 세포의 이동과 면역세포의 발달을 조절하는 기능을 한다. 특히, 암 조직에서는 암세포의 발달과 전이에 밀접한 연관이 되어있어 많은 연구가 진행되고 있다. CXCR4는 정상조직에서의 중요한 역할 때문에 직접적인 조절은 많은 부작용을 초래하기 때문에 CXCR4의 직접적인 저해는 임상 적용의 한계가 있다. 따라서 정상 조직에는 영향을 주지 않고 암 조직의 CXCR4를 특이적으로 조절 할 수 있는 방법에 대한 연구가 필수적이다. 최근 연구에 따르면 GPCR이 많은 경우 단일 수용체로 작용하기 보다는 복합체를 형성하여 보다 다양한 기능을 하고 있다는 결과가 밝혀지고 있다. 이를 통해 본 실험에서는 이분자 형광 상보 기법과 Co-internalization 분석을 통해 CXCR4와 상호작용하는 GPCR을 확인하고, CXCR4의 여러 신호 전달

방식 중 고리형에이엠피와 칼슘을 매개하여 전달하는 것이 이형이량체화를 통한 간접적인 조절이 가능하다는 것을 밝힘으로써, CXCR4를 목적으로 하는 약물 개발연구에 새로운 방향과 신약재창출의 가능성을 제시 할 수 있다.

주요어: CXCR4, 이형이량체화, 신약재창출, 암