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EFFECT OF LONG-TERM NITRIC OXIDE EXPOSURE ON H460 LUNG CANCER CELL MOTILITY

Miss Arpasinee Sanuphan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
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Thesis Title	EFFECT OF LONG-TERM NITRIC OXIDE EXPOSURE ON H460 LUNG CANCER CELL MOTILITY
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Thesis Advisor	Assistant Professor Pithi Chanvorachote, Ph.D.

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ไนตริกออกไซด์ที่พบในบริเวณโดยรอบของเซลล์มะเร็งปอดอาจมีบทบาทสำคัญต่อพฤติกรรมของเซลล์มะเร็ง เพื่อศึกษาผลกระทบที่อาจเป็นไปได้ของไนตริกออกไซด์ต่อพฤติกรรมการเคลื่อนที่ของเซลล์มะเร็งปอด งานวิจัยนี้จึงทำการทดลองโดยให้เซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็ก เอช 460 สัมผัสกับไนตริกออกไซด์โดยใช้สารให้ไนตริกออกไซด์ชนิด dipropylenetriamine (DPTA) NONOate ในความเข้มข้น 0, 5, และ 10 μM ซึ่งไม่เป็นพิษต่อเซลล์มะเร็งเป็นระยะเวลา 0 - 14 วัน และตรวจวิเคราะห์พฤติกรรมการเคลื่อนที่ของเซลล์มะเร็งปอด ผลการศึกษาพบว่าเมื่อเซลล์มะเร็งปอดได้รับไนตริกออกไซด์เป็นระยะเวลานาน 7 วัน และ 14 วัน จะมีพฤติกรรมการเคลื่อนที่เพิ่มขึ้นอย่างมีนัยสำคัญ เมื่อเทียบกับเซลล์มะเร็งปอดในกลุ่มควบคุมที่ไม่ได้รับไนตริกออกไซด์ ทั้งนี้การเคลื่อนที่ของเซลล์มะเร็งปอดจะแปรผันตรงตามความเข้มข้นของสารให้ไนตริกออกไซด์และแปรผันตรงตามระยะเวลาที่เซลล์มะเร็งปอดได้รับไนตริกออกไซด์อีกด้วย เมื่อศึกษากลไกระดับโมเลกุลทำให้ทราบว่าการเคลื่อนที่ของเซลล์ที่เพิ่มขึ้นนั้นมีความสัมพันธ์กับการแสดงออกที่เพิ่มขึ้นของโปรตีน caveolin-1 (Cav-1) ซึ่งเหนี่ยวนำให้เกิดการเพิ่มระดับของโปรตีน focal adhesion kinase (FAK) และ ATP-dependent tyrosine kinase (Akt) ที่อยู่ในรูปพร้อมทำงาน การเพิ่มขึ้นของทั้งโปรตีน phosphorylated FAK และ phosphorylated Akt ทำให้เซลล์มะเร็งปอดมีการเคลื่อนที่เพิ่มขึ้น นอกจากนี้ยังพบด้วยว่าเมื่อเซลล์มะเร็งปอดได้รับไนตริกออกไซด์จะมีจำนวน filopodia ต่อเซลล์เพิ่มขึ้น โดยการเพิ่มขึ้นของ filopodia สอดคล้องกับระดับโปรตีน cell division cycle 42 (Cdc42) ที่เพิ่มขึ้น ผลการทดลองจากงานวิจัยครั้งนี้แสดงให้เห็นว่าเมื่อเซลล์มะเร็งปอดได้รับไนตริกออกไซด์เป็นระยะเวลานานจะกระตุ้นพฤติกรรมการเคลื่อนที่ของเซลล์ผ่านทางโปรตีน Cav-1 และการค้นพบนี้ทำให้ช่วยเสริมสร้างความรู้ความเข้าใจในชีววิทยาของโรคมะเร็งมากยิ่งขึ้น ซึ่งอาจเป็นประโยชน์ในการค้นคว้าหาเป้าหมายใหม่ๆ ในการออกฤทธิ์ของยาต้านมะเร็งต่อไป

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556

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KEYWORDS : NITRIC OXIDE/ LONG-TERM/ METASTASIS/ MIGRATION/ LUNG CANCER

ARPASINEE SANUPHAN : EFFECT OF LONG-TERM NITRIC OXIDE EXPOSURE ON H460 LUNG CANCER CELL MOTILITY.
ADVISOR: ASSIST. PROF. PITHI CHANVORACHOTE, Ph.D., 68 pp.

Nitric oxide (NO) found in the vicinity of lung cancer cells may play a role in the regulation of cancer cell behaviors. To explore the possible effects of NO on cell motility, human lung cancer cells H460 were exposed to non-toxic concentrations of NO donor, dipropylenetriamine (DPTA) NONOate, (0, 5, and 10 μM) for 0-14 days and the migratory characteristics of the cells were determined. The present study found that long-term treatment with NO significantly enhanced cell migration in a dose- and time-dependent manner. Furthermore, we found that the increased migratory action was associated with the increased expression of caveolin-1 (Cav-1), which in turn activated the focal adhesion kinase (FAK) and ATP-dependent tyrosine kinase (Akt) pathways. Notably, the NO-treated cells exhibited an increased number of filopodia per cell, as well as an increase in the levels of cell division cycle 42 (Cdc42) protein. Together, these results indicate that extended NO exposure has a novel effect on cell migration through a Cav-1-dependent mechanism, a finding that strengthens our understanding of cancer biology. In addition, the insights from this study may aid the discovery of novel molecular targets for anti-cancer strategies.

Department : Pharmacology and Physiology Student's Signature .....
Field of Study : Pharmacology Advisor's Signature .....
Academic Year : 2013 .....

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LIST OF ABBREVIATIONS

%	=	percentage
^{o}C	=	degree Celsius
µg	=	microgram
µL	=	microliter
µM	=	micromolar
Akt	=	ATP-dependent tyrosine kinase
ANOVA	=	analysis of variance
BSA	=	bovine serum albumin
Cav-1	=	caveolin-1
Cdc42	=	cell division cycle 42
CO_2	=	carbon dioxide
DMSO	=	dimethylsulfoxide
DPTA	=	dipropylenetriamine
et al.	=	et alii, and others
FAK	=	focal adhesion kinase
FBS	=	fetal bovine serum
h	=	hour
HRP	=	horseradish peroxidase
IU	=	international unit
min	=	minute (s)
mL	=	milliliter

MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	=	nanometer
NO	=	nitric oxide
p-Akt	=	phosphorylated Akt
PBS	=	phosphate-buffered saline
p-FAK	=	phosphorylated-FAK
RPMI	=	Roswell Park Memorial Institute
SRB	=	sulforhodamine B
TBS	=	tris-buffered saline
TBST	=	tris-buffered saline with 0.1% Tween

CHAPTER I

INTRODUCTION

The cancer microenvironment has been reported to have a significant impact on cancer cells in many ways (Hanahan and Weinberg, 2000). Indeed, in such an active environment, cell signaling molecules as well as mediators including pro-inflammatory cytokines, reactive species, and so on, are found to be intensified (Manda, Nechifor, and Neagu, 2009). Among them, the concentrations of nitric oxide (NO), a reactive nitrogen specie synthesized by many cells, such as endothelial, immune, and tumor cells, are found to be dramatically increased in lung cancer environments (Lala, and Chakraborty, 2001; Keibel, Singh, and Sharma, 2009). Excessive and uncontrolled NO production is associated with the pathogenesis of lung cancer (Masri, 2010). Additionally, clinical observation has shown that NO levels in the lungs of lung cancer patients were increased in comparison to those of normal subjects (Masri *et al*., 2005; Esme *et al*., 2008). While cytokines have been shown to have significant effects on the behavior of cancer cells within microenvironment, the effects of long-term nitric oxide exposure on lung cancer cell motility remain unknown.

The ability of cancer cells to migrate is an important hallmark of successful metastasis (Geho *et al*., 2005). The metastasis cascade is a multistep process that consists of five components: local migration and invasion, intra-vasation, circulation, extra-vasation, and colony formation at secondary sites (Mina and Sledge, 2011). Tumor cells need to be motile to invade tissues; this motility is achieved by changing their cell–cell adhesion properties and by reorganizing their cytoskeletons. These

cellular mechanisms are regulated by various signaling molecules, including the Rho family of small GTPases, caveolin-1 (Cav-1), and focal adhesion kinase (FAK) (Nobes and Hall, 1995; Parri and Chiarugi, 2010). FAK is activated by an initial autophosphorylation at the Tyr 397 residue, and its activation is essential for the regulation of focal adhesion turnover and cell protrusion (Cary, Chang, and Guan, 1996; Serrels *et al*., 2007). Studies have reported that FAK mediates cells motility through the activation of the downstream Akt signaling pathway (Satyajit, Daniel, and David, 2005). Furthermore, evidence has suggested that Cdc42 overexpression increased cell motility by inducing the formation of filopodia (Nobes and Hall, 1995; Allen *et al*., 1997; Kaibuchi, Kuroda, and Amano, 1999). Recently, Caveolin-1 (Cav-1), a 21-24 kDa integral membrane protein, has garnered increasing attention as its role in the regulation of cancer cell behaviors has been revealed (Terence and Michael, 2005; Chanvorachote *et al*., 2009; Luanpitpong *et al*., 2010; Rungtabnapa *et al*., 2011; Pongjit and Chanvorachote, 2011; Chunhacha *et al*., 2012; Songserm, Pongrakhananon, and Chanvorachote, 2012; Halim, Luanpitpong, and Chanvorachote, 2012; Suchaoin and Chanvorachote, 2012; Chunhacha and Chanvorachote, 2012). Increased Cav-1 expression was shown to be associated with enhanced progression of prostate, colon, and breast cancers (Ho *et al*., 2002; Chunhacha and Chanvorachote, 2012). Likewise, elevated Cav-1 expression was associated with an increased metastasis capacity and poor survival in lung cancer patients (Chunhacha and Chanvorachote, 2012; Sotgia *et al*., 2012). We investigated the role of long-term exposure to non-toxic doses of NO on lung carcinoma cell motility and examined the possible underlying mechanisms using pharmacological approaches. The findings of

the present study aid in the better understanding of this microenvironment-related mediator and may help in the development of novel anti-cancer strategies.

Research Questions

1. What are the effects of long-term NO exposure on H460 lung cancer cell migration?
2. What are the underlying mechanisms of long-term NO exposure in regulation of H460 lung cancer cell migration?

Hypothesis

Long-term NO exposure enhances migration of H460 lung cancer cells through the induction of migration-related proteins including Cav-1, p-FAK, FAK, p-Akt, Akt, and Cdc42.

Objectives

1. To investigate the effect of long-term NO exposure on migratory activities of H460 lung cancer cells.
2. To study mechanisms regarding migration potentiating effect of long-term NO exposure in H460 lung cancer cells.

CHAPTER II

LITERATURE REVIEW

Lung cancer

Lung cancer remains the major cause of cancer death worldwide (Jemal *et al*., 2010). The lung cancer deaths are predicted to account for 27% of the estimated 228,190 cancer deaths diagnosed in 2013 (American Cancer Society, 2013). Despite advances in early detection as well as standard treatment, such as surgery, chemotherapy, and radiation, lung cancer mortality rate still remains high (Spiro and Silvestri, 2005). The main risk factor for lung cancer is cigarette smoking, accounting for about 0.8% of all cancer deaths (Navada *et al*., 2006; American Cancer Society, 2013). In addition, genetic and environmental factors also contribute to gene mutation (Anand *et al*., 2008). Apparently, p53 and epidermal growth factor receptor (EGFR) gene mutation in lung cancer lead to the activation of cell division and inhibition of apoptosis (Herbst, Heymach, and Lippman, 2008).

Generally, lung cancer is divided into two main types called small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). About 15% of all lung cancers are small cell lung cancer (SCLC). Compared to another types of cancer, SCLC can grow rapidly and be able to metastasize to distant sites contributing to poor prognosis (Sher, Dy, and Adjei, 2008). The treatment of SCLC that has spread can prolong life for 6 - 12 months (Samson *et al*., 2007). Moreover, SCLC tends to increase at a greater rate of metastasis compare to the other types of lung tumor.

Nevertheless, SCLC has more responsive to cytotoxic chemotherapy and radiation therapy compare to NSCLC (Timbrell, 2008).

On the other hand, NSCLC is the most common type of lung cancer accounting approximately 85% of the total lung cancer cases (American Cancer Society, 2013). NSCLC is classified into 3 common sub-types, namely squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (Travis *et al*., 2004). NSCLC tends to grow and metastasize slower than SCLC. The early lung cancer may not show any symptoms until the disease is advanced (Muers, Holmes, and Littlewood, 1999). Treatment for NSCLC depends on different types and stages of lung cancer. Hence, screening and early detection of lung cancer is beneficial to the outcome of treatment. Nevertheless, the 5-year survival rates for all stages of lung cancer remained low at 16 % in 2002-2008 (American Cancer Society, 2013).

Nowadays, there are only a few types of cancer that can be detected in its early stage by chemotherapy, radiation therapy or surgery at the origins of organs (Jemal *et al*., 2011). The cancer cells may spread to nearby locations of the body without being detected which will not be cured by surgery (American Cancer Society, 2012). However, over the past 20 years there have been many improvements in the treatment of lung cancer such as radiation treatment, technology in diagnostic imaging, and new chemotherapy drugs. Therefore, the development of new chemotherapy drugs that have specific molecular targets is receiving more and more attention (West *et al*., 2012).

Metastasis

About 90% of deaths in cancer patients are associated with cancer metastasis (Mehlen and Puisieux, 2006). Metastasis occurs when the cancer cells metastasize or spread from the site of its original site to another part of the body (Chiang and Massagué, 2008). Generally lung cancer is likely to metastasize via the lymphatic and hematogenous routes to various organ of the body, but it is most often spread to the adrenal glands (35% of total cases), liver (30% to 45% of total cases), bones (25% to 40% of total cases), and brain (10% of total cases) (Veerappan, Lettieri, and Cuneo, 2003).

The metastasis cascade is a multistep process consisting of five steps: local migration, intravasation, circulation, extravasation, and colony formation at secondary sites (Mina and Sledge, 2011). The processes of metastasis are as follow. A cancer cells must first detach from the primary tumor, then invade the surrounding tissue, after that penetrate into lymphatic and/or blood vessels. Next the detached cell must be able to survive in the circulatory system and arrest in capillaries or venules of other organs then leave the bloodstream, and lastly form the new tumor at the secondary site (Bacac and Stamenkovic, 2008; O'Hayre *et al*., 2008) (as shown in Figure 2.1).

Each of these processes is controlled by distinct molecular pathways leading to enhanced migratory properties and cytoskeletal changes (Legate, Wickström, and Fässler, 2009; Bravo-Cordero, Hodgson, and Condeelis, 2012). Therefore, a better understanding the molecular mechanisms of cancer metastasis is required to help

researchers and clinicians to develop effective prevention, diagnosis, and treatment for cancer disease (Perlikos, Harrington, and Syrigos, 2013).

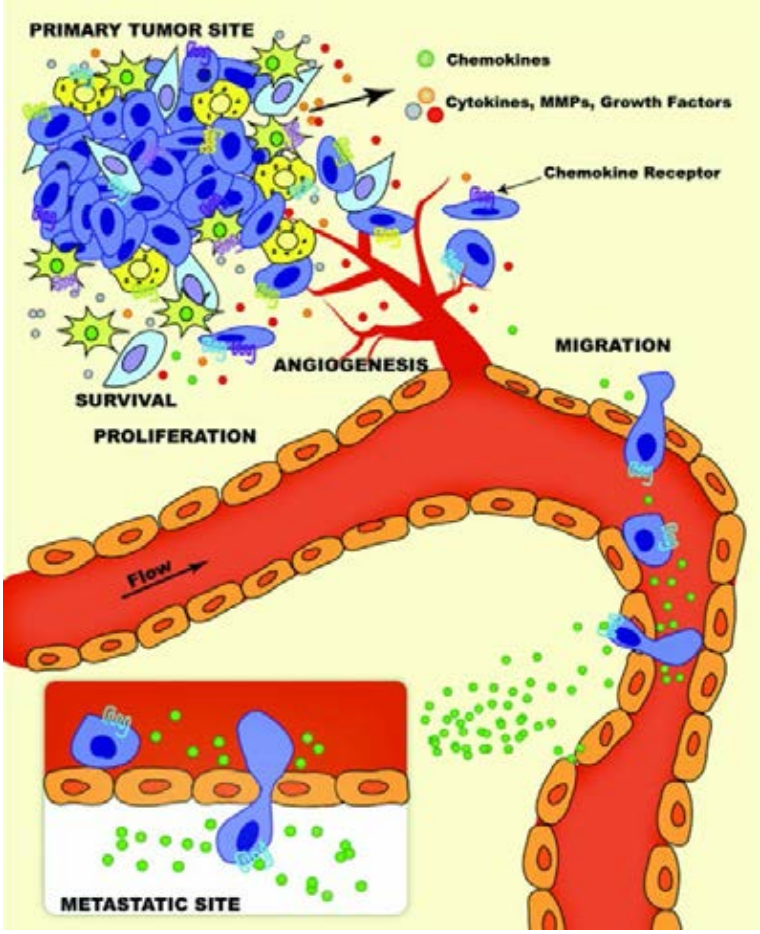


Figure 2.1 Principal steps in metastasis (O'Hayre *et al*., 2008)

Migration

Cell migration is a critical parameter in metastasis, the leading cause of cancer deaths (Yilmaz and Christofori, 2010). Morover, cell migration is essential for normal biological processes, such as the development of an organism (morphogenesis), wound healing, angiogenesis, and immune response (Friedl and Wolf, 2009).

Cancer cells have the ability to migrate and invade the surrounding tissue allowing them to undergo positional changes within the tissues, which may cause cancer metastasis (Hanahan and Weinberg, 2000; Friedl and Wolf, 2003). Therefore, migration is the pivotal parameters in the metastatic cascade as well as the leading cause of cancer death (Friedl and Wolf, 2003; Bacac and Stamenkovic, 2008).

Many studies have shown that the regulation of focal adhesion complexes occur during cell migration (Kim and Wirtz, 2013). Focal adhesions are multi-protein complexes. These adhesion complexes are associated with the linkage between actin cytoskeleton and extracellular matrix (ECM) via integrin (Burridge *et al*., 1988) (as shown in Figure 2.2).

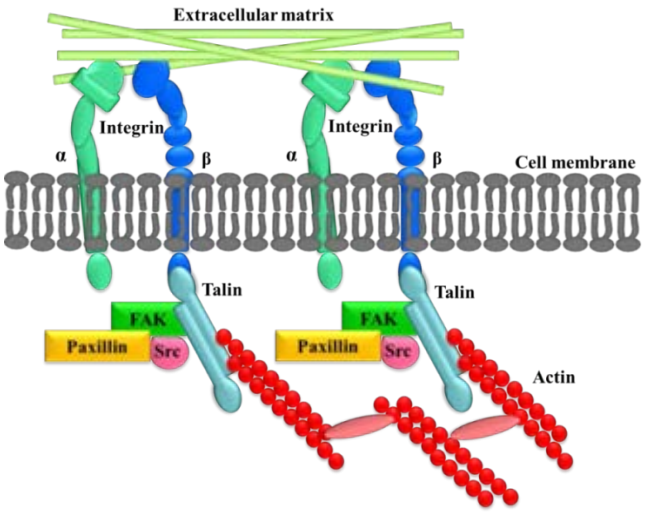


Figure 2.2 Schematic drawing of focal adhesion site

The focal adhesion is mediated by integrins that bind to ECM and convert extracellular signals into cells via the interaction with intracellular molecules such as focal adhesion kinase (FAK) and actin filaments of the cytoskeleton (Wozniak *et al*.,

2004). Thus, cell migration plays a crucial role in an early step of the progression of cancer, and up regulation of proteins that related to cell migration in tumor cells (Bogenrieder and Herlyn, 2003).

Molecular mechanism of cell migration

In the present study, we have focused on four proteins, including caveolin-1 (Cav-1), focal adhesion kinase (FAK), ATP-dependent tyrosine kinase (Akt) and cell division cycle protein 42 (Cdc42). These proteins play an important role in the control of cell migration.

▪ Caveolin-1

Caveolae are plasma membrane flask-shaped lipid rafts with enriched cholesterol and sphingolipids, that contain the marker protein namely caveolin (Fujimoto *et al*., 2000; Pike, 2006). The key components of caveolae are the caveolins. The caveolin gene family consists of caveolin-1, caveolin-2 and caveolin-3. These proteins are essential for the formation of caveolae as well as the regulation of signaling molecules (Fujimoto *et al*., 2000). The expression of caveolin-1 and caveolin-2 can be found in adipocytes, endothelial cells and fibroblasts (Razani and Lisanti, 2001). For the expression of caveolin-3, it can only be found in skeletal muscle, cardiac cells and diaphragm (Song *et al*., 1996).

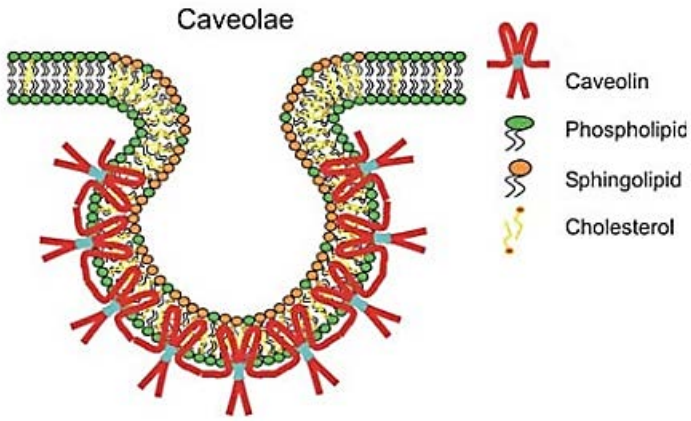


Figure 2.3 Structure of caveolae (Razani and Lisanti, 2002)

The main coating protein of caveolae is caveolin-1 (Cav-1), a 21-24 kDa integral membrane protein with both the amino terminus and carboxy terminus directed toward the cytoplasm (Lisanti *et al*., 1995). Cav-1 interacts with numerous signaling molecules, including the non-receptor tyrosine kinases, PI-3 kinase, and eNOS (Liu *et al*., 1997).

Caveolin-1 was implicated in the regulation of cell migration (Grande-Garcı´a and del Pozo, 2008). Generally, the expression of Cav-1 is associated with many cellular processes, such as vesicular transport, cholesterol homeostasis, cell migration, and cellular transformation (Fujimoto *et al*., 2000; Pike, 2006). In addition, Cav-1 is phosphorylated at tyrosine residue 14 by the Src, Fyn and Abl tyrosine kinases (Sanguinetti and Mastick, 2003; Sanguinetti, Cao, and Mastick, 2003). Likewise, phosphorylation of this residue can regulate signal transduction, endocytosis and cell migration (Sanguinetti and Mastick, 2003; Sanguinetti, Cao, and Mastick, 2003).

Moreover, the phosphorylation of Cav-1 at Tyr 14 that is important to stabilize the localization of FAK within the focal adhesion (FA) site causing an enhanced turnover of focal adhesion and increased tumor cell migration and metastasis (Fabry *et al*., 2011; Nethe and Hordijk, 2011). On the other hand, the knockdown of Cav-1 in non-small cell lung cancer cells lead to reduced phospho-focal adhesion kinase levels (Sunaga *et al*., 2004)

In addition, several studies revealed that Cav-1 can regulate cell migration through a PI3K/Akt-dependent mechanism (Ravid *et al*., 2005; Li *et al*., 2009) consistent with previous report showing the overexpression of Cav-1 leads to induced of Akt activation (Chanvorachote *et al*., 2009). As knockdown of Cav-1 affects Akt activity by reduced level of the pAkt level when compared with control cells (Luanpitpong *et al*., 2010).

Furthermore, the expression of Cav-1 has been observed in various types of cancer, associating with cancer cells invasive capacity by promoting metastasis leading to poor survival of patients, such as lung, breast, pancreas, esophagus, thyroid, prostate and colon cancers (Ho *et al*., 2002; Chunhacha and Chanvorachote, 2012; Sotgia *et al*., 2012). Additionally, many studies have reported on the roles of Cav-1 on tumor progression, such as induction of angiogenesis, influence cancer cell migration, resistance to apoptosis, and multidrug resistance (Hanahan and Weinberg, 2000). Thus, Cav-1 expression may have prognostic significance in various types of cancer with an emphasis on its variable roles in metastasis and tumor progression (Hanahan and Weinberg, 2000; Chunhacha and Chanvorachote, 2012).

▪ Focal adhesion kinase (FAK)

Focal adhesion kinase (FAK) is a 125 kDa non-receptor tyrosine kinases, which consists of a central kinase domain flanked by an N-terminal FAK-erzrin-radixin-moesin (FERM) domain, a C-terminal domain containing a proline-rich motifs (Pro-1 and Pro-2) and a focal adhesion targeting (FAT) domain (Li and Hua, 2008; Zhao and Guan, 2011) (as shown in Figure 2.4).

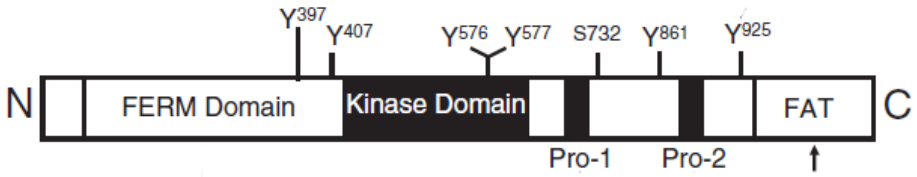


Figure 2.4 The Schematic of focal adhesion kinase domain structure with phosphorylation sites (Zhao and Guan, 2011)

In addidtion, FAK can be activated by phosphorylation, which the FERM domain and the kinase domain blocks FAK autophosphorylation at tyrosine (Y) 397 site (Schaller *et al*., 1995). The autophosphorylation can occur through binding of the FERM domain and integrin and subsequently SH2-domain of Src kinase bind to Y397 site (Schaller *et al*., 1995). Thus, downstream targets can be phosphorylated by the FAK/Src kinase complex (Lietha *et al*., 2007). Furthermore, growth factors, cytokines or clustering of integrins helps the rapid phosphorylation of FAK at tyrosine (Y) 397 site (Schaller, 2001).

FAK is present in focal adhesions. Many reports showed that the recruitment and activation of FAK at focal adhesion sites lead to downstream responses as well as

plays a role in cell survival, cell growth and migration (van Nimwegen and van de Water, 2007). Likewise, the overexpression of FAK was found in different cancer types, such as oral, pancreatic, kidney, lung, brain, melanoma, head and neck, thyroid, ovarian, and colon cancer as well as its expression related with tumor progression (Owens *et al*., 1995; Hao *et al*., 2009).

FAK knockdown leads to decreased expression of FAK expression results in cell migration defects (Schlaepfer and Mitra, 2004). On the other hand, increased FAK expression is a hallmark of many cancers and may contribute to the metastatic phenotype of fully malignant cells (Gabarra-Niecko *et al*., 2003).

- **ATP-dependent tyrosine kinase (Akt)**

Akt or protein kinase B (PKB) is a serine/threonine protein kinase consisting of three isoforms, Akt1, Akt2 and Akt3. It plays an essential role in cell survival, cell proliferation, apoptosis, angiogenesis and chemotaxis (Testa and Bellacosa, 2001; Bellacosa, Testa, and Larue, 2004).

Moreover, Akt1 and Akt2 are found in all types of tissue, however testinal and neural tissue also express of Akt3. Akt can be activated by phosphatidylinositol 3-kinase (PI3K) as well as PI3K/Akt signaling which are associated with the activation of integrin and regulation of actin reorganization (Testa and Bellacosa, 2001; Bellacosa, Testa, and Larue, 2004; Rigor *et al*., 2009). Interestingly, the overexpression of the PI3K/Akt signaling pathway was found to be related to several types of cancer, such as ovarian and colon cancer (Golubovskaya, Kweh, and Cance, 2009). A recent study reported an Akt expression in non-small cell lung cancer

(NSCLC) cells which may play an important role in developing malignant phenotype of cancer cells (Lee *et al*., 2011). In addition, AKT is a downstream signal of FAK pathway, thus AKT and FAK are attractive therapeutic targets for cancer treatment.

- **Cell division cycle protein 42 (Cdc42)**

The cell division cycle 42 (Cdc42), a member of the Rho family small GTPases, is able to switch between an active form (GTP-bound) and inactive form (GDP-bound) by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Whitehead, Zohn, and Der, 2001) (Figure 2.5). Cdc42 facilitates activation of various downstream cascades leading to cytoskeleton remodeling in tumor cell, which causes alterations in cellular activities, such as cell motility and metastasis. (Sinha and Yang, 2008).

Many studies have been reported that Cdc42 plays an important role in regulation of metastasis (Gupta and Massagué, 2006) by promoting polymerization and organization of actin filaments of tumor cells (Jaffe and Hall, 2002). In eukaryotic cells can be found actin filaments, which filamentous actin (F-actin) has a role in many cellular processes such as cell migration (Jaffe and Hall, 2002).

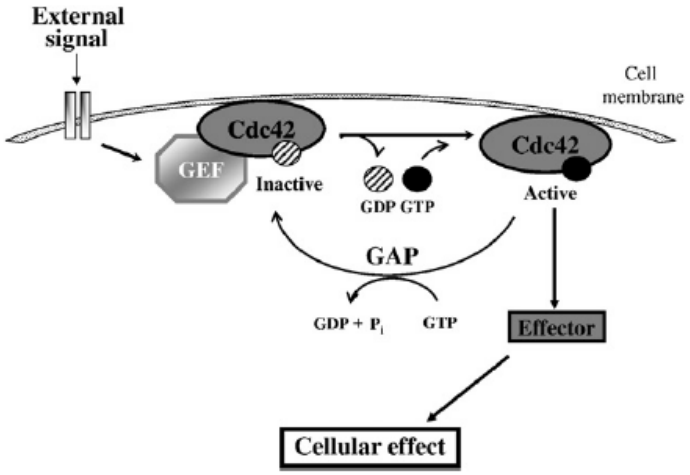


Figure 2.5 Activation of Cdc42 (Sinha and Yang, 2008)

Moreover, F-actin cytoskeleton is regulated by Cdc42 which controls the protrusion of cell, namely filopodia (Nobes and Hall, 1995) (as shown in Figure 2.6). Cellular morphology changes during the migration through actin polymerization at cell membrane leading to formation of filopodia or cell protrusions (Mattila and Lappalainen, 2008). Therefore the onset of cell motility is indicated by observation of filopodia.

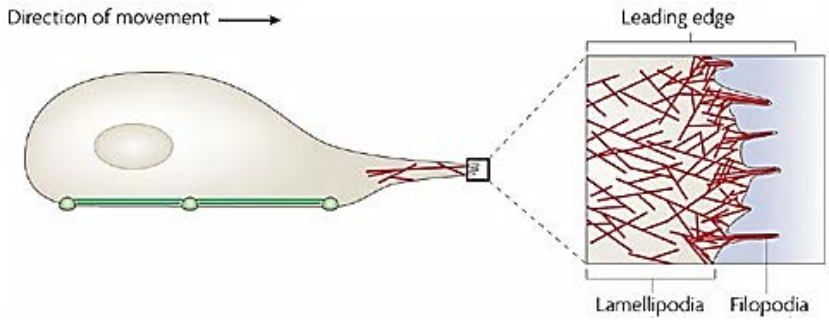


Figure 2.6 Both lamellipodia and microspikes are motile structures regulated by Rho proteins (Mattila and Lappalainen, 2008)

In addition, the overexpression of Cdc42 is found in several types of human cancer cells comparing to normal tissue (Fritz, Just, and Kaina, 1999; Sahai and Marshall, 2002; Kamai *et al*., 2004). Also the density of filopodia found to be increase (Arjonen, Kaukonen, and Ivaska, 2011). This leads to a suggestion that Cdc42 may be a key protein driving carcinogenesis and tumor progression (Gupta and Massagué, 2006).

The tumor microenvironment

A recent study found that the malignant phenotype of tumor not only depend on the intrinsic characteristics of tumor cells, but it also influenced by the cooperative interactions of the components in the tumor microenvironment (Cretu and Brooks, 2007; Whiteside, 2008). The tumor microenvironment complex can be divided into 4 groups (Cretu and Brooks, 2007):

1. Cancer cells
2. Non-cancer cells (inflammatory cells, immune cells)
3. Secreted soluble factors (growth factors, interleukins)
4. Non-cellular solid material (extracellular matrix or ECM)

As the cancer progresses, the tumor microenvironment can become activated by cell signaling molecules as well as mediators including pro-inflammatory cytokines and reactive species. These mediators play an important role in tumor progression and metastasis (Balkwill and Mantovani, 2001; Manda, Nechifor, and Neagu, 2009). Moreover, several studies suggested that tumor microenvironment has

been associated with chronic inflammatory conditions (Balkwill and Mantovani, 2001; Grivennikov, Greten, and Karin, 2010).

Furthermore, the epidemiological studies have shown that about 15–20% of all deaths from cancer were associated with chronic inflammation in different types of cancer. Most cases of lung cancer are highly associated with chronic inflammation (Balkwill and Mantovani, 2001). The identification of the molecular pathways involved in cancer-related inflammation may help in development of diagnosis and treatment of cancer (Mantovani *et al*., 2008).

Nitric Oxide

Both oxidative and reductive stresses play a significant role in the progression of cancer (Acharya *et al*., 2010). Likewise, nitric oxide and reactive nitrogen species are essential in physiological and pathological processes (Moncada, Palmer, and Higgs, 1991). The variable effects of nitric oxide (NO) in tumors may depend on the localization of NO synthase and its activity, the concentration and duration of NO exposure, and the cellular sensitivity to NO (Lala and Chakraborty, 2001; Keibel *et al*., 2009; Masri, 2010).

Nitric oxide is generated from the transformation of amino acid L-Arginine to L-citrulline by nitric oxide synthase (NOS) (Thomsen *et al*., 1995). NADPH (reduced nicotinamide adenine dinucleotide), oxygen, and other cofactors with heme, tetrahydrobiopterin, flavin nonucleotide, and flavin adenine dinucleotide, are necessary in this reaction (Thomsen *et al*., 1995; Xu *et al*., 2002).

There are three isoforms of NOS, namely, NOS1 (neuronal NOS or nNOS), NOS2 (inducible NOS or iNOS) and NOS3 (endothelial NOS or eNOS) (Thomsen *et al*., 1995). NOS1 is found in brain and spinal cord. NOS2 is found in lymphocytes, neutrophils, eosinophils, and macrophages existed in the spleen, lungs and colon. NOS3 is found in endothelial cells. NOS1 and NOS3 are constitutively Ca^{2+}-calmodulin (CaM) dependent. On the other hand, NOS2 is Ca^{2+}- CaM independent. The activation of NOS2 requires regulatory cytokines including interferon gamma and tumor necrosis factor (Knowles and Moncada, 1994; Thomsen *et al*., 1995; Xu *et al*., 2002).

Figure 2.7 Nitric Oxide synthetic

Role of nitric oxide in migration

Cell migration is an essential for many physiological and pathological processes. For example, in immune system, several immune cells have to migrate from their origin to antigens (e.g. virus, bacteria, or parasite) or to other sites of the

body. NO has been shown to influence cellular motility (Isenberg, 2003; Secco *et al*., 2003). A recent study reported that when human umbilical vein endothelial cells (HUVEC) were incubated with an immune complex and then treated with L-arginine, transendothelial migration was significantly increased (Isenberg, 2003). In addition, cancer cell migration is a key component in metastasis which NO has been shown to be highly involved with the migratory ability of cancer cells (Woodhouse, Chuaqui, and Liotta, 1997). For example, mammary adenocarcinoma cells showed positive correlation between NO and migration of cancer cells (Ridnour *et al*., 2008).

Migration in cancer cells may be enhanced by disrupting cell barrier causing the release of endogenous NO (Xu, Lu, and Deitch, 2002). For example, the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) can increase the permeability of polarized enterocyte monolayers which causes dilation of tight junctions between enterocytes (Salzman *et al*., 1995; Xu *et al*., 2002). Hence, the NO production during migration can help cancer cells to migrate from primary tumor site to secondary sites.

Nitric oxide and lung cancer

Within a cancer microenvironment, NO can be synthesized by many cells, such as endothelial, immune, and tumor cells (Manda *et al*., 2009). All three NOS isoforms were expressed in many different types of cancer (Lala and Chakraborty, 2001; Keibel *et al*., 2009). Furthermore, in lung cancer tissue, an increased expression of iNOS was correlated with tumor progression (Kan *et al*., 2004). A recent study found that cigarette smoking induced the production of iNOS, which may contribute to the growth and progression of lung cancer cell (Chen *et al*., 2008). Additionally,

clinical observation has shown that NO levels in the lungs of lung cancer patients were increased in comparison to those of normal subjects (Masri *et al*., 2005; Esme *et al*., 2008). However, there is no report on the relationship between expression of NO and the biology of the tumors in human (Radosevich *et al*., 2009).

In addition, a recent study has focused on NO concentration and how it correlates with molecular mechanisms of cell proliferation and death by co-culturing activated macrophages with MCF-7 breast cancer cells at varying ratios (Ridnour *et al*., 2008). However, the threshold of NO concentration may vary in different tumors. Moreover, both the microenvironment of the tumor and the spatial and temporal delivery of the NO are believed to play key roles in interfering cellular pathways (Wink and Mitchell, 1998). Thus, the chronic inflammation is associated with the development of cancer which NO may have a role in these processes (Mantovani *et al*., 2008; Whiteside, 2008).

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and Reagents

The NO donor dipropylenetriamine (DPTA) NONOate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phalloidin tetramethylrhodamine B isothiocyanate, sulforhodamine B (SRB), bovine serum albumin (BSA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibodies consist of Cav-1, p-Akt (S473), Akt, p-FAK (Y397), FAK, Cdc42, β-actin, and secondary antibody conjugated to horseradish peroxidase (HRP) were obtained from Cell Signaling (Danvers, MA, USA). Lipofectamine 2000 and PrestoBlue were obtained from Invitrogen (Carlsbad, CA, USA).

2. Instruments

- Automated cell counter (TC10^{TM} Bio-Rad Laboratories, Inc., USA)
- Autopipette: 0.2-2 µL, 2-20 µL, 20-200 µL and 100-1,000 µL (Gilson, Inc., France)
- Cell culture plate: 6-well and 96-well (Corning Inc., USA)
- Centrifuge (Z 383 Hermle labortechnik, Germany)
- Fluorescence microplate reader (SpectraMax® M5, Molecular Devices, USA)
- Fluorescence microscope (IX51 with DP70 Olympus, Japan)
- Laminar flow cabinet and humidified incubator (Bosstech scientific instruments, USA)

- Microplate reader (Anthros, Durham, NC, USA)
- Mini Trans-Blot® cell and PowerPac™ Basic Power Supply (Bio-Rad Laboratories, Inc., USA)
- Nitrocellulose membranes (Bio-Rad Laboratories, Inc., USA)
- pH meter (CG 842 Schott instruments, Germany)
- Vortex mixer (Vortex-Genie® 1, Scientific Industries, USA)

3. Sample preparation

Nitric Oxide at various concentrations was solubilized in phosphate buffered saline (PBS).

4. Cell culture

Human non-small cell lung cancer cells (NCI-H460) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, MD, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

For long-term NO exposure experiment, cells were cultured in medium containing NO donor dipropylenetriamine (DPTA) NONOate (0, 5, and 10 μM) for 7 and 14 days, respectively. Briefly, the cells were subcultured (passaged) into the culturing medium containing freshly prepared NO donor every three day (as shown in figure 3.1). After the H460 lung cancer cells were exposed to NO donor (0, 5, and 10

μM) for 7 and 14 days, the cells would be subjected to the experiments without any more adding of NO donor.

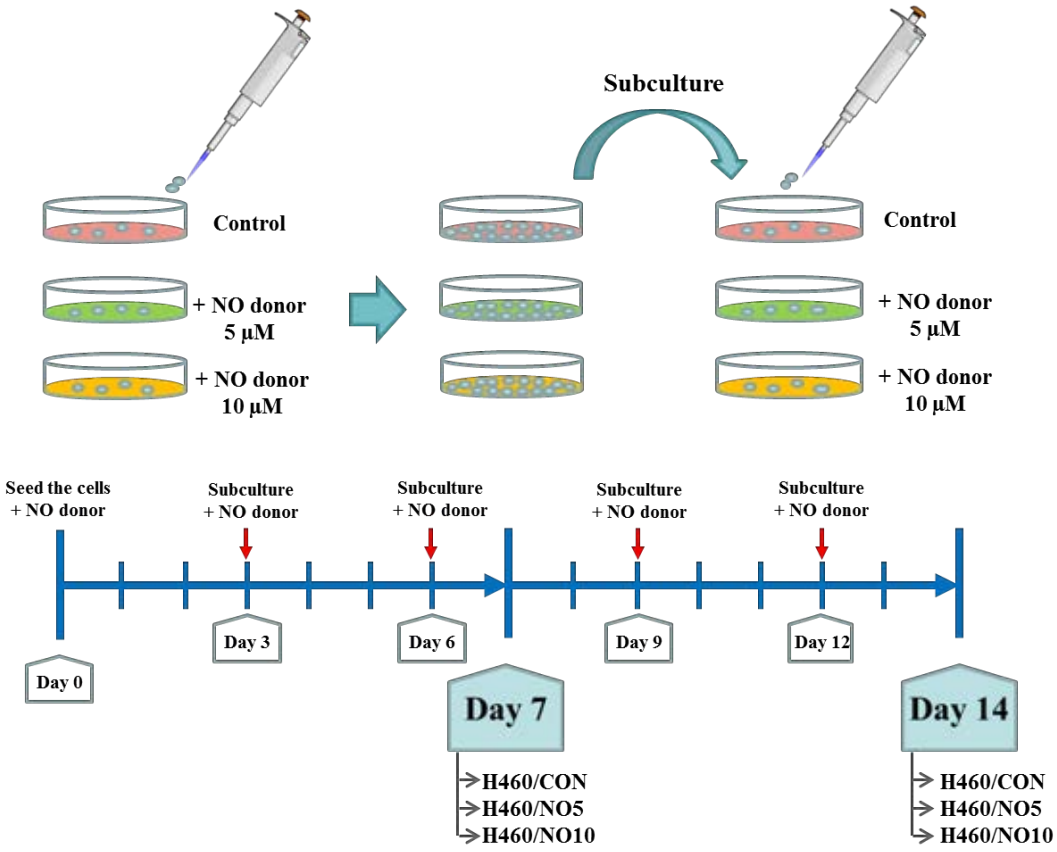


Figure 3.1 Schematic illustration and timeline for long-term NO donor exposure experiment

5. Experimental design

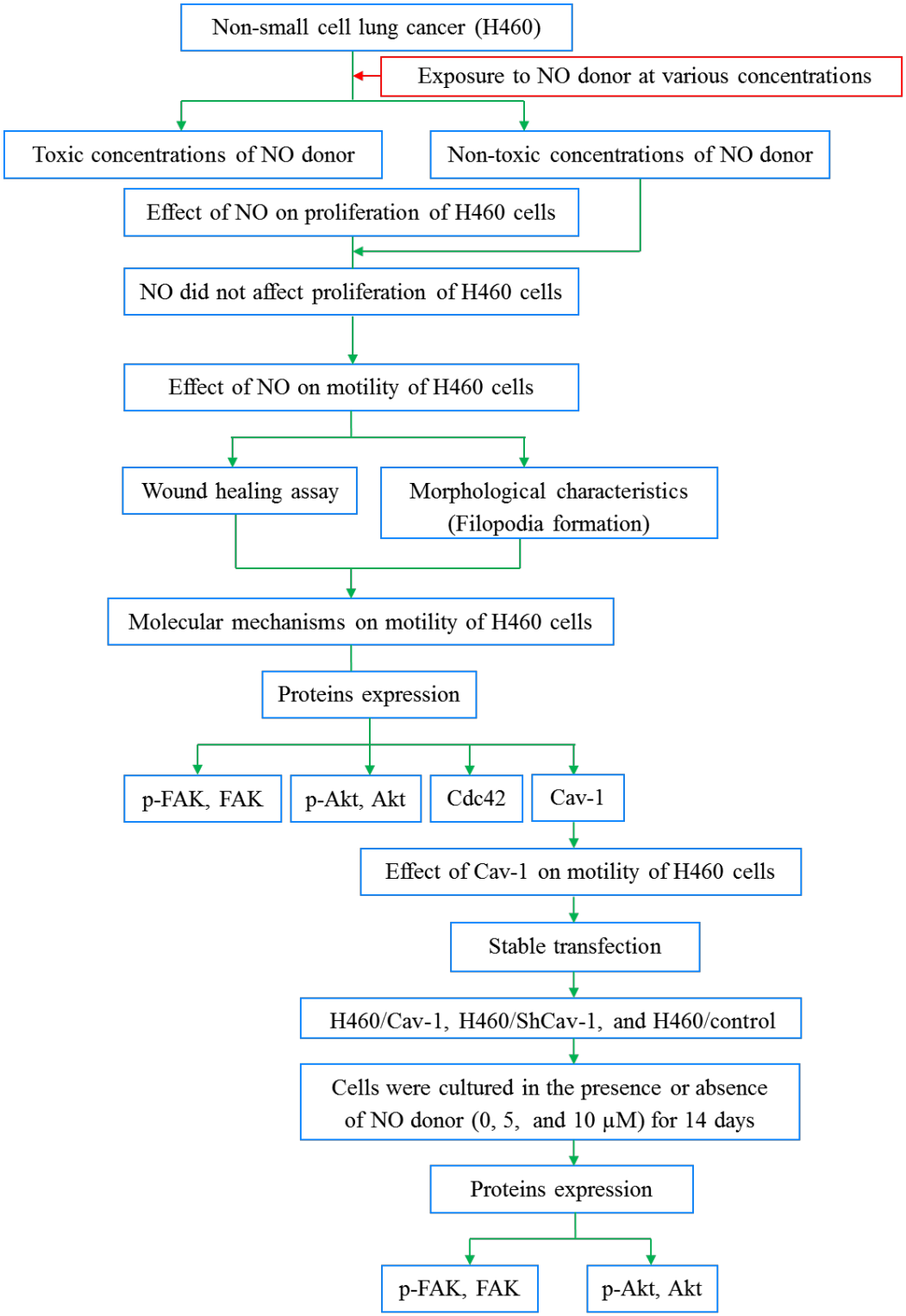


Figure 3.2 Experimental designs of this study

5.1 Effect of NO donor on cells viability of H460 lung cancer cells

To investigate the concentrations of NO donor that did not effect on H460 cells viability, cell viability was determined using the MTT assay that the numbers of live cells based on activity of the mitochondrial reductase enzyme. Cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate and exposed to the NO donor at various concentrations (0 - 20 μM) and incubated for 12 h. Then, cells were treated with MTT (5.0 mg/mL in PBS) and incubated for 4 h at 37°C. Then, the MTT solution was removed and 100 μL DMSO was added to dissolve the formazan crystal. The intensity of the formazan product was measured at 570 nm using a microplate reader (Anthros, Durham, NC, USA). The percentage of cell viability was calculated using the following formula:

Cell viability (%) = (A_{570} of treatment × 100) / A_{570} of control.

**A_{570} = absorbance at 570 nm

5.2 Effect of NO donor on proliferation of H460 lung cancer cells

To investigate effect of NO donor on cell proliferation, cell proliferation was determined using PrestoBlue, which is a resazurin based compound, membrane permeable solution, and non-toxic to the cells, which the proliferation of cells can be defined as the number of live cells based on living cells reduce resazurin by mitochondrial reductase into resorufin then the solution changed into red. Cells were seeded at a density of $5×10^3$ cells/well in

a 96-well plate and exposed to the NO donor at various concentrations (0, 5, and 10 μM) and incubated for 0, 24, and 48 h. Then, cell viability was examined by cell proliferation assay. Cell proliferation was determined through incubation with PrestoBlue at a 1:10 dilution for 1 h, and the fluorescence intensity of the resazurin product (resorufin) was measured at 530 nm (excitation wavelength) and 590 nm (emission wavelength) using a fluorescence microplate reader (SpectraMax® M5, Molecular Devices, USA).

5.3 Effect of the long-term NO exposure on cell migration and of H460 lung cancer cells

To investigate the effect of long-term NO exposure on cell migration, the migration ability of H460 cells was evaluated using wound-healing assay. Cells were exposed to the NO donor at various concentrations (0, 5, and 10 μM) and incubated for 7 or 14 days. Then, cells were subjected and seeded at a density of $2x10^4$ cells/well in a 96-well plate. Cells were grown to a confluent monolayer in a 96-well plate, and then a scrape was made down the center of the well using a P200 micropipette tip. The well was then rinsed with phosphate-buffered saline (PBS) and replaced with RPMI medium. At the indicated times (0, 12 and 24 h), the wound spaces were imaged under a phase-contrast microscope (10X) (Olympus IX51 with DP70), and the wound spaces were measured on the image field at four points per field. The percentage change in the wound space at time 12 and 24 h was calculated using the following formula:

Space change at time 12, 24 h (%) =

$$\frac{\text{(Average space at time 0 h) - (Average space at time 12, 24 h)}}{\text{(Average space at time 0 h)}} \times 100$$

Relative cell migration was calculated by dividing the percentage change in the wound space of treated cells by that of the control cells in each experiment.

5.4 Effect of the long-term NO exposure on cell morphology of H460 lung cancer cells

To investigate the effect of long-term NO donor exposure on cell morphology, cell morphology was investigated using phalloidin-rhodamine and sulforhodamine B staining assays. Cells were exposed to the NO donor at various concentrations (0, 5, and 10 μM) and incubated for 7 or 14 days. Then, cells were subjected and seeded at a density of $2x10^3$ cells/well in a 96-well plate and incubated for 24 h. After NO exposure, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at 37°C, permeabilized with 0.1% Triton-X100 in PBS for 4 min, rinsed with PBS, and then blocked with 0.2% BSA for 30 min. The cells were then incubated with either a 1:100 dilution of phalloidin-rhodamine in PBS or 0.4% sulforhodamine B in 1% acetic acid for 15 min; the cells were then rinsed 3 times with PBS and mounted with 50% glycerol. The cell morphology was imaged using a fluorescence microscope (Olympus IX51 with DP70).

5.5 Effect of the long-term NO exposure on the expression of the migration-related proteins in H460 lung cancer cells

To investigate the underlying mechanism of NO donor enhanced H460 cells migration, cells were exposed to the NO donor at various concentrations (0, 5, and 10 μM) for 7 or 14 days and analyzed by Western blotting. The expression levels of the migration-related proteins, namely Cav-1, p-FAK, FAK, p-Akt, Akt, and Cdc42, were evaluated. After NO donor exposure, cells were incubated with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche Molecular Biochemicals) for 30 min on ice. The cell lysates were collected, and determined for protein content using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein from each sample (40 μg) were denatured by heating at 95°C for 5 min with Laemmli loading buffer and loaded onto 10% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked in 5% non-fat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, and 0.05% Tween 20) for 30 min and incubated with the appropriate primary antibodies overnight at 4°C. Membranes were washed three times with TBST for 10 min and incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h at room temperature. The immune complexes were detected by chemiluminescence (Supersignal West Pico; Pierce, Rockfore, IL, USA) and quantified using analyst/PC densitometry

software (Bio-Rad). To confirm equal loading of the samples, the blots were reprobed with β-actin antibody.

5.6 Effect of the long-term NO exposure on the expression of the migration-related proteins through Cav-1-dependent mechanism in H460 lung cancer cells

To investigate whether the up-regulation of p-FAK, FAK, p-Akt, and Akt proteins was mediated through Cav-1-dependent mechanism, the expression profile of these proteins after overexpression or downregulation of Cav-1 was determined. The Cav-1 expression plasmid was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and the Cav-1 short hairpin knockdown plasmid (shRNA-Cav-1) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stable transfection of cells with the Cav-1 expression plasmid or the Cav-1 knockdown plasmid was achieved by culturing the cells until they reached approximately 60% confluence. Then, 15 μL Lipofectamine 2000 reagent and 2 μg Cav-1 expression plasmid, shRNA-Cav-1, or control plasmid were used to transfect the cells in the absence of serum. After 12 h, the medium was replaced with fresh culture medium containing 5% FBS. Approximately 36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin, and the cell suspensions were plated in 75 mL culture flasks and cultured for 20 to 30 days with antibiotic selection. The stable transfectants were pooled, and the expression of the Cav-1 protein in the transfectants was confirmed by Western

blotting. The cells were cultured in antibiotic-free RPMI 1640 medium for at least two passages before experiments were performed.

The Cav-1 overexpressed cells (H460/Cav-1) and Cav-1 knockdown cells (H460/ShCav-1) were cultured in the presence or absence of NO donor (0, 5, and 10 μM) for 14 days, then cells were collected and protein expression was identified by Western blotting.

6. Statistical Analysis

The mean data from independent experiments were normalized to the results of the control cells. The values are presented as the mean ± standard deviation (SD) from three or more independent experiments and were analyzed using one-way ANOVA with a post-hoc test (Tukey's test) at a significance level of $P < 0.05$ using SPSS version 16.0.

7. Conceptual framework

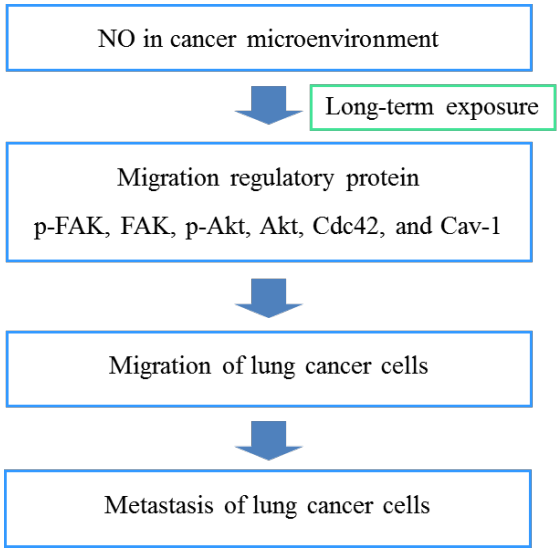


Figure 3.3 Conceptual frame work of this study

CHAPTER IV

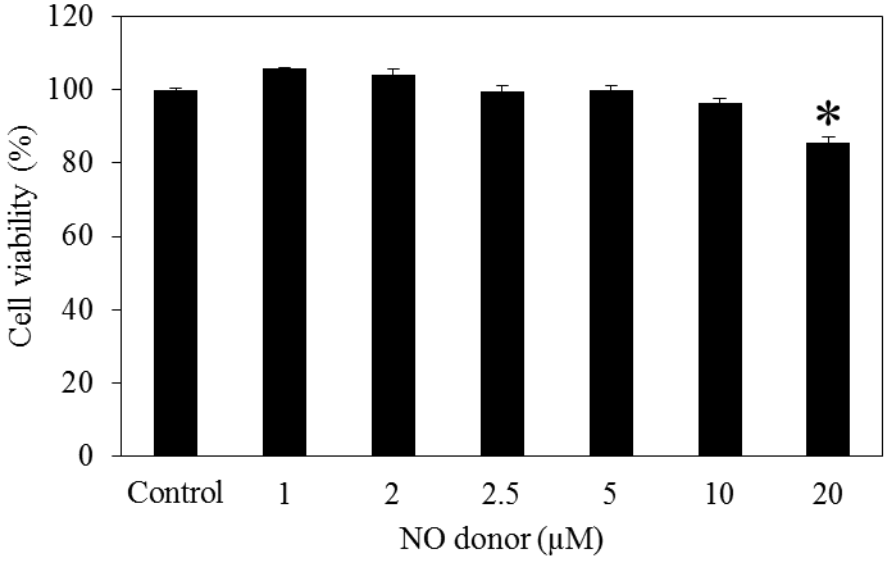
RESULTS

1. Effect of NO donor on the viability of H460 lung cancer cells

We first characterized the effects of NO donor on the viability of H460 lung cancer cells. The H460 cells were cultured in the presence or absence of DPTA NONOate (1-20 μM), a slow-releasing NO donor compound, for 24 h, and cell viability was determined using the MTT assay. This assay is based on the activity of the mitochondrial reductase enzyme in live cells. Figure 4.1(A) shows that when cells were treated with the NO donor, at concentrations ranging from 1-10 μM, neither cytotoxicity nor proliferative effects were observed in the cells. A significant decrease in viability was first detected in cells treated with 20 μM DPTA NONOate; however, approximately 90% of the cells still remained viable.

To investigate the effect of long-term NO treatment on cell proliferation, cells were cultured in their optimal conditions supplemented with 5 or 10 μM NO donor and were subjected to the cell proliferation assay for 0, 24, 48 h. Then, their proliferative behavior was evaluated using cell proliferation assay. Cell proliferation was determined through incubation with PrestoBlue, which is a resazurin based compound, membrane permeable solution, and non-toxic to the cells. The proliferation of cells can be defined as the number of live cells based on living cells reduce resazurin by mitochondrial reductase into resorufin resulting in color change of the solution into red. As Figure 4.1(B) indicates, the NO-treated cells exhibited no significant changes in cell proliferation during the test period.

(A)



(B)

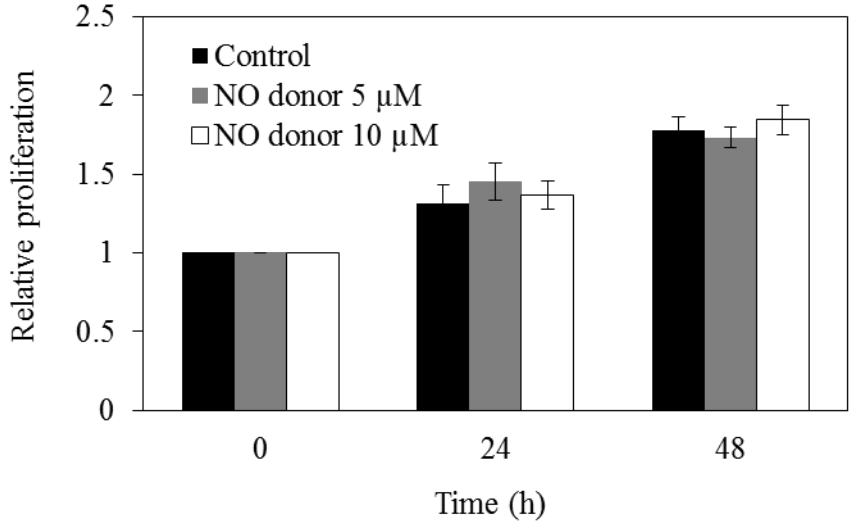
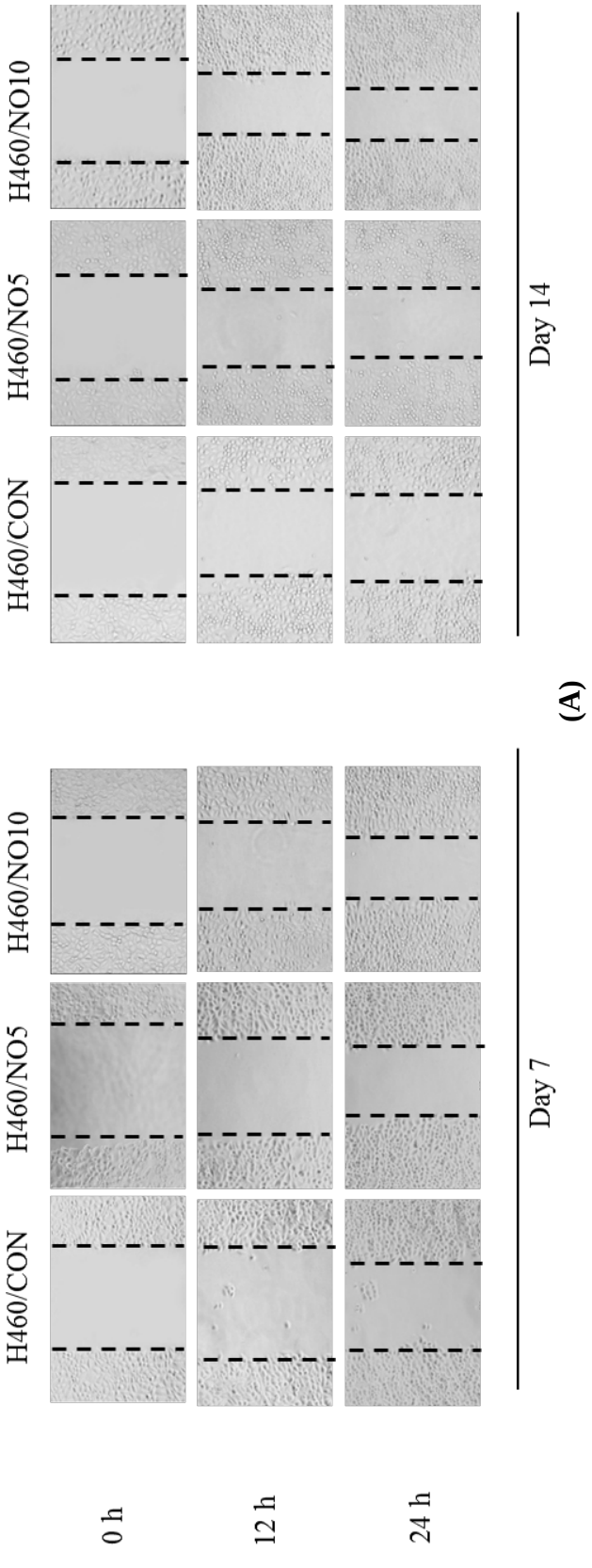


Figure 4.1 Effect of NO donor on cytotoxicity in lung carcinoma H460 cells. (A) Effect of DPTA NONOate on H460 cell viability. H460 cells were treated with various concentrations (0-20 μM) of DPTA NONOate for 24 h. The cell viability was analyzed using the MTT assay. (B) Proliferative effect of DPTA NONOate on H460

cells. Cell proliferation for 24 and 48 h was determined using PrestoBlue. The data are the mean ± S.D. (n = 3). * $P < 0.05$ versus the non-treated control.

2. Long-term NO exposure potentiates migration of H460 cells

To investigate the effect of NO donor on cell migration, we performed scratch wound-healing assays. Cells were exposed to NO for 7 or 14 days and were subjected to the migration assay for 12 and 24 h (as shown in Figure 4.2(A)). Figures 4.2(A) and 4.2(B) show that long-term treatment with the NO donor significantly enhanced the motility of the cells in dose- and time-dependent manners as compared with the H460 control cells. Treatment with 10 μM DPTA NONOate for 14 days potentiated the migration of the cells approximately 2.5-fold as compared with the non-treated cells, as shown in Figure 4.2(B).


H460/CON
H460/NO5
H460/NO10
H460/CON
H460/NO5
H460/NO10
0 h
12 h
24 h
Day 7
Day 14

(A)

(B)

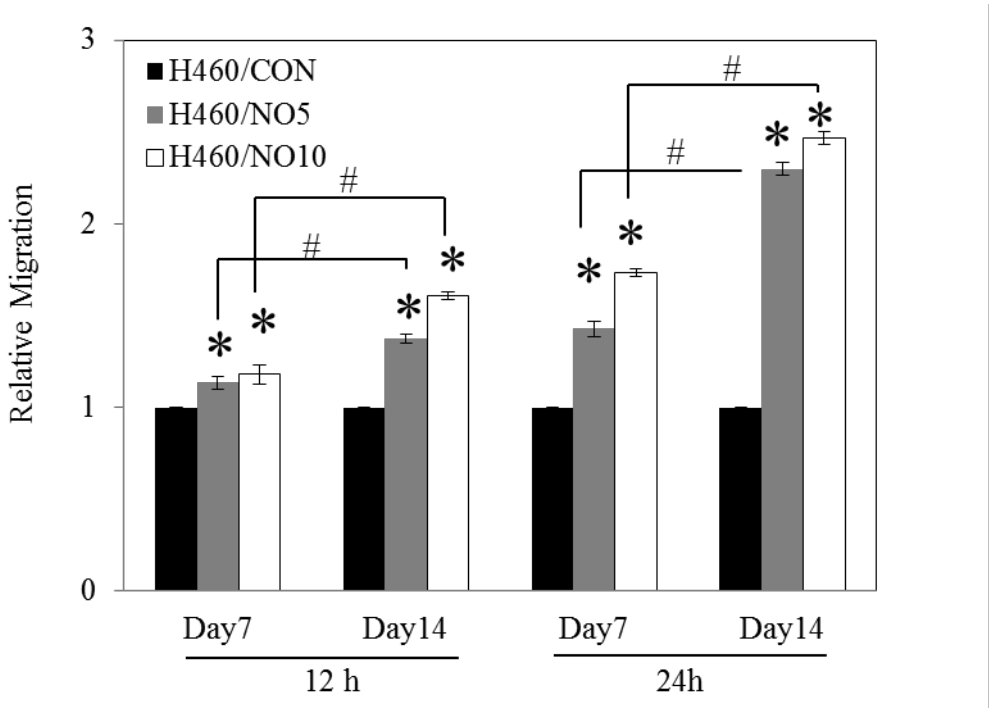


Figure 4.2 Effect of nitric oxide exposure on H460 cell migration. (A) Cells were exposed to NO donor at various concentrations for 7 or 14 days and subjected to a migration assay. Phase-contrast images were captured at 0, 12 and 24 h. (B) The relative cell migration was determined by comparing the relative change in wound space to the control cells. The data are the mean ± S.D. (n = 3). * $P < 0.05$ versus the control cells. # $P < 0.05$ versus NO-treated cells for 7 days.

3. NO enhances filopodia formation in lung cancer cells

Filopodia are generated through actin polymerization and rearrangement of actin filaments, and the formation of filopodia has been linked to increased tumor cell migration. To evaluate the effect of NO treatment on filopodia formation, cells were exposed to the NO donor at various concentrations (0, 5, and 10 μM) and incubated for 7 or 14 days. The presence of filopodia was determined using a phalloidin-rhodamine staining assay. In addition to this staining, the cytoskeletal actin was also stained with sulforhodamine B dye. Figures 4.3(A) and 4.3(B) indicate that when H460 cells were cultured in the presence of the NO donor, the cells exhibited an altered actin alignment and an increased number of filopodia.

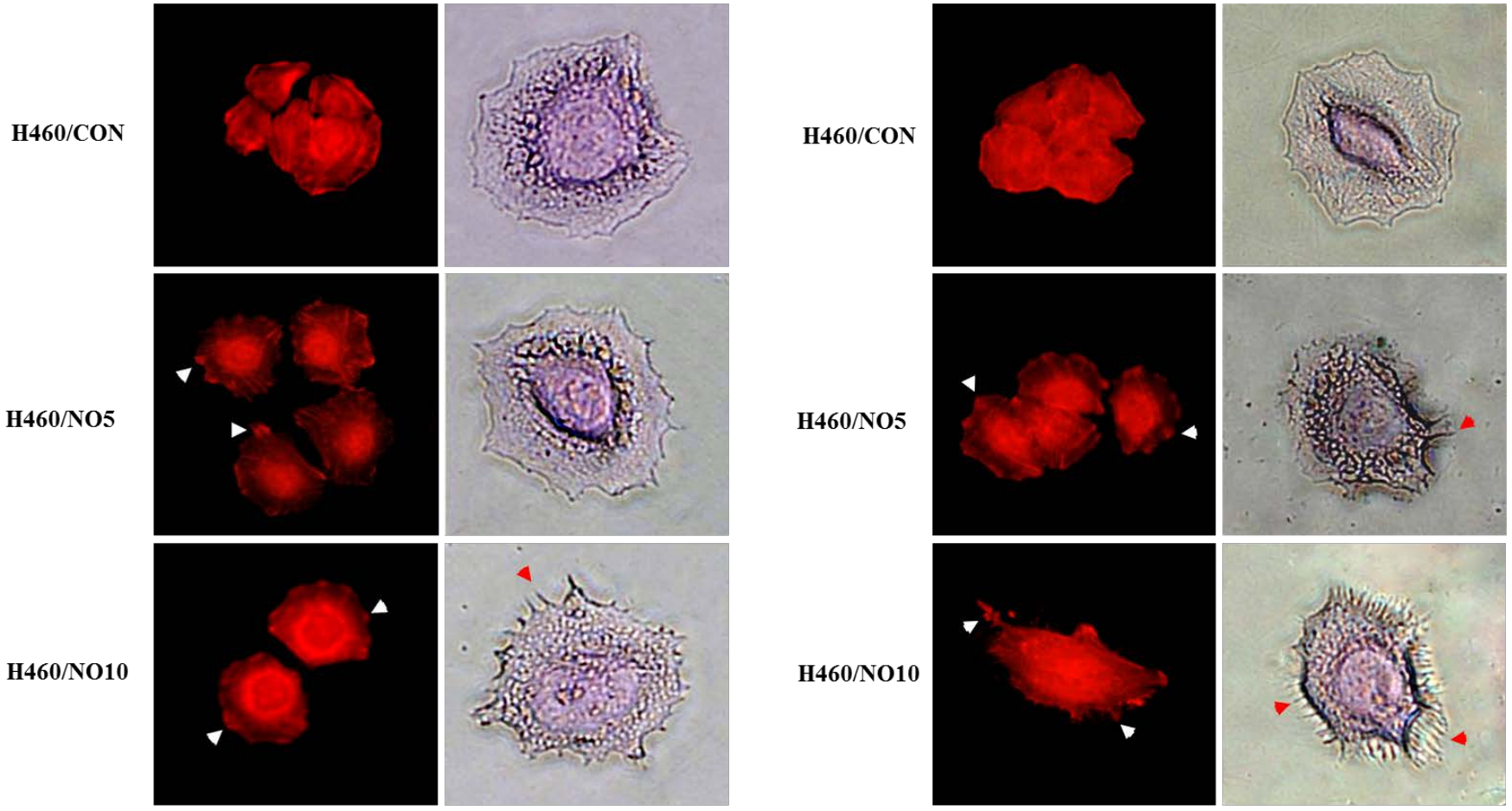
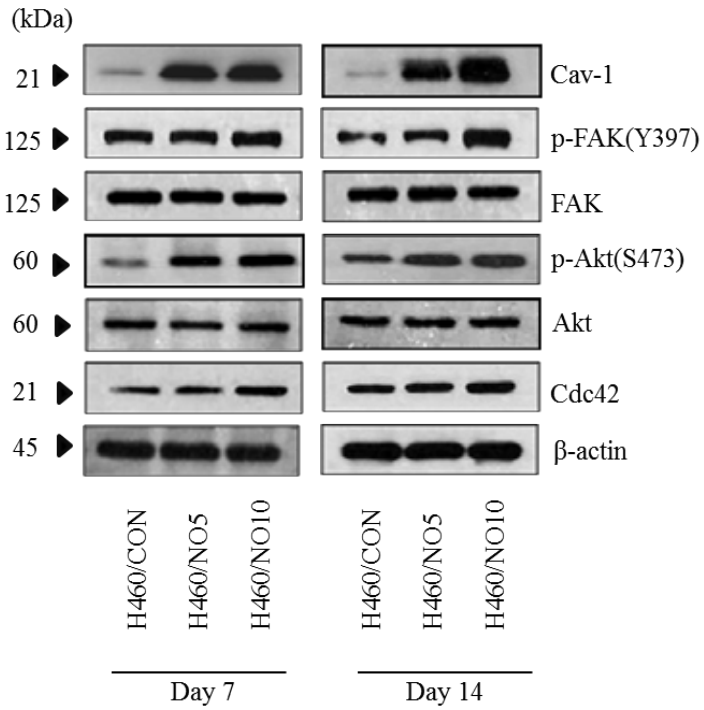

H460/CON
H460/NO5
H460/NO10
Day 7
(A)
H460/CON
H460/NO5
H460/NO10
Day 14
(B)

Figure 4.3 Filopodia formation in H460 cells treated with nitric oxide. H460 cells were treated with NO donor at concentrations of 0, 5, and 10 μM for (A) 7 days and (B) 14 days. The cells were then stained with phalloidin-rhodamine and sulforhodamine B dye.

4. The long-term NO exposure induces Cav-1-dependent FAK and Akt activation

Having demonstrated the potentiating effect of NO exposure on lung cancer cell motility, we next examined the underlying mechanism, focusing on the expression levels of the proteins known to play roles in cell migration. Cancer cells were treated with NO donor at different concentrations for 7 and 14 days, and were analyzed by western blot. Expression levels of the migration-related proteins, namely Cav-1, FAK, Akt, and Cdc42, were evaluated. Figures 4.4(A) and 4.4(B) show that NO exposure for 7 and 14 days significantly increased the levels of Cav-1, phosphorylated FAK (Tyr 397), phosphorylated Akt (Ser 473), and Cdc42, whereas NO exposure had no significant effect on the levels of total FAK and total Akt. Interestingly, the effects of NO on the mentioned proteins appeared to be dose- and time-dependent; cells treated with 10 μM NO donor for 14 days exhibited the most pronounced changes in protein levels as compared to cells treated with 5 μM NO donor or cells that were treated for a shorter period of time.

(A)


(kDa)
21
125
125
60
60
21
45
Cav-1
p-FAK(Y397)
FAK
p-Akt(S473)
Akt
Cdc42
β-actin
H460/CON
H460/NO5
H460/NO10
H460/CON
H460/NO5
H460/NO10
Day 7
Day 14

(B)

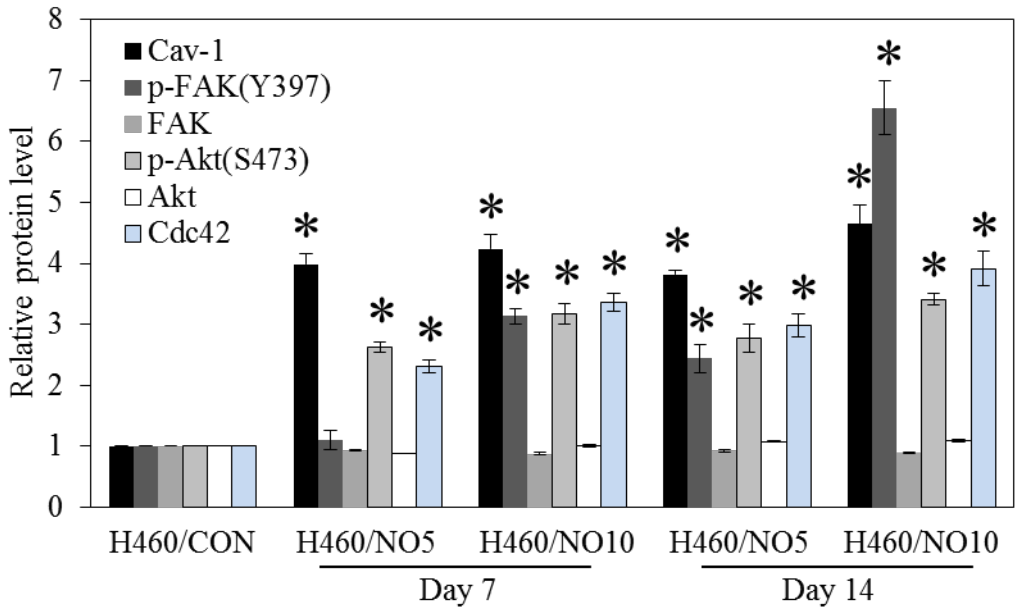
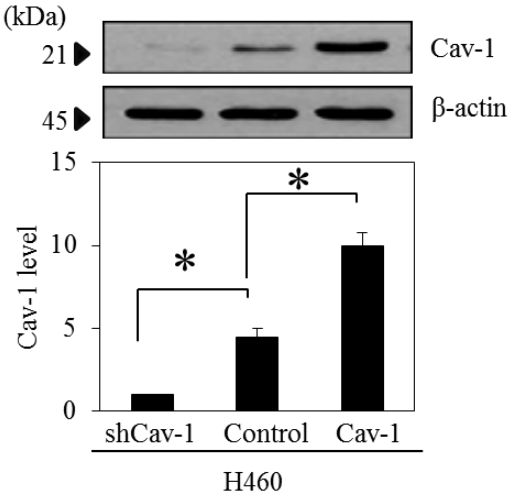

Relative protein level
0
1
2
3
4
5
6
7
8
Cav-1
p-FAK(Y397)
FAK
p-Akt(S473)
Akt
Cdc42
H460/CON
H460/NO5
H460/NO10
H460/NO5
H460/NO10
Day 7
Day 14

Figure 4.4 Nitric oxide exposure activates the FAK-Akt pathways. (A) NO-treated cells at 7 and 14 days were subjected to Western blotting, and the expression of phosphorylated FAK, total FAK, phosphorylated Akt, total Akt, Cdc42, and Cav-1 were determined. To confirm equal loading of the samples, the blots were reprobed with β-actin antibody. (B) The immunoblot signals were quantified by densitometry. The data are the mean ± S.D. (n = 3). * $P < 0.05$ versus the non-treated control.

As Cav-1 has been shown to function as an adaptor protein that regulates the activities of other proteins as previously described (Pongjit and Chanvorachote, 2011), we tested whether the up-regulation of the proteins mentioned above was through Cav-1-dependent mechanism. Using gene manipulation approaches, Cav-1 overexpressed and knockdown cells were generated as described in Materials and Methods. As expected, western blot analysis of Cav-1 expression showed a substantial increase in Cav-1 protein level in the Cav-1-transfected cells, whereas a significant decrease in Cav-1 level was observed in the shRNA-Cav-1-transfected cells as compared with the control-transfected cells (Figure 4.5(A)). The Cav-1 over expressing cells (H460/Cav-1), the Cav-1 knockdown cells (H460/ShCav-1), and the control H460 cells were cultured in the presence or absence of NO (5-10 μM) for 14 days, and the levels of phosphorylated FAK (Tyr 397), phosphorylated Akt (Ser 473), and their total protein levels were determined. Figure 4.5(B) shows that the Cav-1 overexpressed cells (H460/Cav-1) exhibited a significantly increased level of phosphorylated FAK and phosphorylated Akt, whereas the total FAK and total Akt levels were not affected. In contrast, the NO-mediated FAK and Akt phosphorylation events were suppressed in the cells in which Cav-1 was knocked down (H460/ShCav-

1 cells). These results indicate that long-term NO exposure in H460 cells induces FAK and Akt activation in a Cav-1-dependent manner.

(A)



(B)

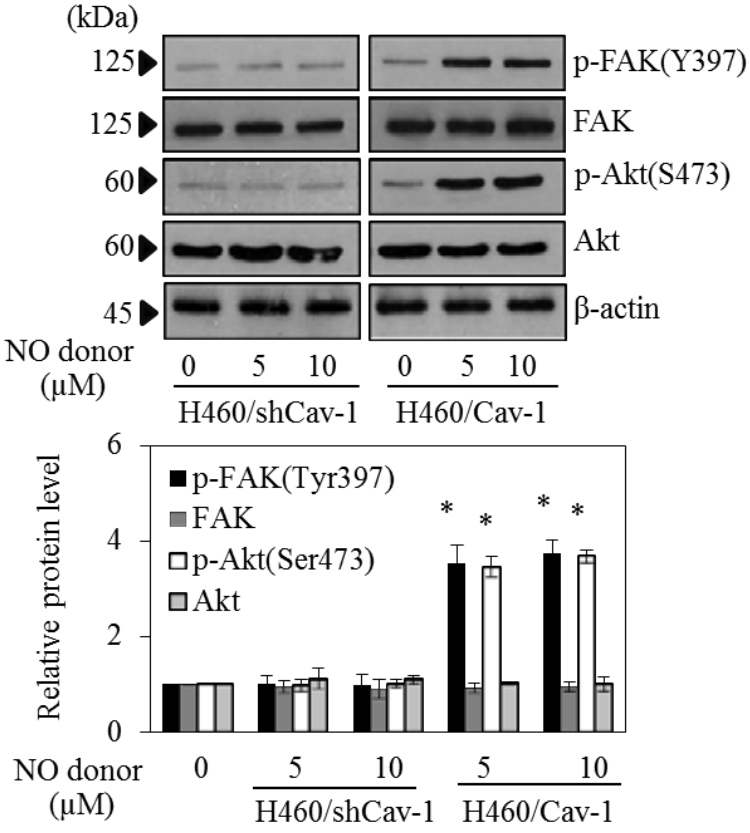


Figure 4.5 Nitric oxide mediated FAK-Akt activation via Cav-1-dependent mechanism. Stable Cav-1 overexpressed (H460/Cav-1) and Cav-1 knockdown (H460/ShCav-1) cell lines were established as indicated in Materials and Methods. (A) The expression level of Cav-1 protein in the control H460, H460/Cav-1, and H460/ShCav-1 cells was determined by Western blotting. (B) The cells were exposed to NO donor for 14 days, and the expression of phosphorylated FAK, total FAK, phosphorylated Akt, and total Akt were determined. The immunoblot signals were quantified by densitometry, and the mean data from the independent experiments were normalized to the control. The data are the mean ± S.D. (n = 3). * $P < 0.05$ versus the control.

CHAPTER V

DISCUSSION AND CONCLUSION

Worldwide, lung cancer is a leading cause of cancer-related death in both men and women (Parkin *et al*., 2002), and approximately 90% of non-small cell lung cancer deaths are attributed to cancer metastasis (Paesmans *et al*., 1995; Sotgia *et al*., 2012). Among the multiple steps of metastasis, migration of the cancer cells has been recognized as an important hallmark for the successful spread of cancer throughout the body (Geho *et al*., 2005; Mina and Sledge, 2011).

Cell migration is regulated by a complex network of signal transduction pathways relating to integrins, kinases, small GTPases, cytoskeletal proteins (Ridley *et al*., 2003; Welf and Haugh, 2011). In addition, cancer cells that undergo a phenotypical change called epithelial to mesenchymal transition (EMT) acquire an increased migratory capability (Kalluri and Weinberg, 2009). EMT is an important process during metastasis of cancer cells by which epithelial cells lose their cell-cell adhesion and gain a migratory phenotype to become mesenchymal cells (Hay, 2005). Therefore, EMT is also a key event in the migration process and is associated with increased metastatic potential of cancer cells (Ishigaki *et al*., 2011).

However, information regarding the key mediators that controls the migratory activities of the cancer cells remains largely unknown. An increase in NO production has frequently been observed in the tissue surrounding the tumor and may be critical for some cancer cells behaviors (Lala and Chakraborty, 2001; Masri *et al*., 2005; Esme *et al*., 2008; Keibel *et al*., 2009; Masri, 2010). In addition, elevated NO

production has been observed in the lung tissue of lung cancer patients in comparison with that of normal subjects (Masri *et al*., 2005; Esme *et al*., 2008). These findings have strengthened the idea that NO presenting in the lung cancer environment may affect the behavior of cancer cells.

NO is a gaseous molecule that is able to diffuse deeply into tissues; indeed, such a substance has been shown to regulate cell behaviors in many ways, including the relaxation of vascular smooth muscle (Moncada and Higgs, 1993; Keibel *et al*., 2009). Controversial roles of NO have been reported for normal cell motility. NO was shown to inhibit vascular smooth muscle cell migration (Moncada and Higgs, 1993; Sarkar *et al*., 1996); however, the opposite effect was observed in the microglia cell model (Moncada and Higgs, 1993; Chen *et al*., 2000). Accordingly, both the inhibitory effect as well as promoting effect of NO on cancer cells have been reported (Chen *et al*., 2000; Dhar *et al*., 2003). The variable effects of NO in tumors may depend on the localization of NO synthase and its activity, the concentration and duration of NO exposure, and the cellular sensitivity to NO (Moncada and Higgs, 1993; Chen *et al*., 2000; Lala and Chakraborty, 2001; Fukumura, Kashiwagi, and Jain, 2006; Keibel *et al*., 2009; Masri, 2010). While long-term effects of NO on lung cancer cell migration are still unknown, Hickok et al. showed that short-term treatment with NO donor for 4, 6, and 24 h inhibited breast cancer cell migration through N-Myc downstream-regulated gene-1 (NDRG1) expression (Dhar *et al*., 2003; Hickok *et al*., 2011). However, in prostate cancer cells, NO was shown to potentiate cell motility (Polytarchou *et al*., 2009; Hickok *et al*., 2011). The present study demonstrated the novel role of long-term NO exposure in the regulation of lung cancer cell migration

that may be important for the fulfillment of cancer insights. Long-term exposure to NO enhances the cells motility via FAK and Akt-dependent mechanisms. In addition, we provided evidence indicating that such an activation of the FAK-Akt pathway is dependent on the level of cellular Cav-1 (as shown in Figure 4.5).

Previous studies found that the phosphorylation of FAK at position Tyr 397 is critical for cell migration (Cary *et al*., 1996; Serrels *et al*., 2007). Furthermore, FAK function on cell motility was shown to involve with its downstream Akt (Satyajit *et al*., 2005; Turecková *et al*., 2009). Our gene manipulation experiments further revealed the role of Cav-1 on FAK-Akt pathway. We found that phosphorylated FAK, as well as phosphorylated Akt, were increased in response to long-term NO treatment of lung cell lines, and this response was limited in the Cav-1 knockdown cells. However, the up-regulation of both phosphorylation events was shown to be intensified in the Cav-1 overexpressed cells (as shown in Figure 4.5). These findings suggest that Cav-1 may have a novel influence on FAK-Akt-mediated cell migration in lung cancer cell models. Cav-1 is the principal component of caveolae membranes. Cav-1 has been reported to promote tumor cell migration and invasion, and an increase in Cav-1 expression is associated with tumor metastasis in lung cancer (Ho *et al*., 2002; Terence and Michael, 2005; Chanvorachote *et al*., 2009; Luanpitpong *et al*., 2010; Rungtabnapa *et al*., 2011; Pongjit and Chanvorachote, 2011; Chunhacha *et al*., 2012; Songserm *et al*., 2012; Halim *et al*., 2012; Suchaoin and Chanvorachote, 2012; Chunhacha and Chanvorachote, 2012). Consistent with its pro-survival role, Cav-1 positively regulated the growth of lung cancer H460 cells when these cells were treated with NO, as previously described (Chanvorachote *et al*., 2009). Since an up-

regulation of NO, as well as Cav-1 protein, is associated with an aggressive status in lung cancer cells, therefore the results from this study may lead to a better understanding of lung cancer pathology.

Likewise, the small GTPase Cdc42 was shown to regulate actin filaments and the migration of tumor cells (Nobes and Hall, 1995; Parri and Chiarugi, 2010). In fibroblasts, Cdc42 induces the rapid formation and extension of filopodia, which are required for movement processes (Nobes and Hall, 1995; Allen *et al*., 1997; Kaibuchi *et al*., 1999). We investigated how NO exposure affected the actin organization in lung cancer cells and found that NO up-regulates Cdc42 protein and enhances the formation of filopodia in these cells.

Nowadays, increasing evidence proposes the functional role of Cav-1 in promoting oncogenic cell transformation, cancer cell migration, tumor progression, and metastasis. Overexpression of Cav-1 has been observed as a marker for tumor progression in many types of cancer such as breast, prostate, and multiple myeloma (Tanase, 2008; Thompson *et al*., 2010). However, further investigations are required to determine the molecular mechanisms of Cav-1 function promote the metastatic progression of cancer (Zhu *et al*., 2004; van Golen, 2006). Therefore, targeting caveolin-1 expression may present a potential novel biomarker that can be used as molecular target in targeted therapy for detection and diagnosis for anti-metastatic therapy in cancer (Zhu *et al*., 2004; Oh *et al*., 2007).

In conclusion, we demonstrated the possible role of long-term NO exposure on the metastatic behaviors of cancer cells, including migration and invasion. NO

exposure activated the FAK-Akt signaling pathway through a Cav-1-dependent mechanism and increased filopodia formation. Elevated NO levels have been observed in cancer environments, thus the knowledge gained from the present study may benefit our understanding of cancer biology and may be useful in the development of cancer therapies.

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APPENDIX

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 1. The percentage of H460 cell viability was evaluated using cytotoxicity assay, after which cells were exposed to NO donor at various concentrations (0 - 20 μM) for 24 h. The data are the mean ± S.D. (n = 3)

NO donor (μM)	Cell viability (% of control)
Control	100.00 ± 0.42
1	105.70 ± 1.31
2	104.00 ± 1.69
2.5	99.40 ± 0.96
5	99.70 ± 0.48
10	96.30 ± 0.35
20	85.40 ± 1.11

Table 2. The relative cell proliferation of H460 cell was evaluated using cell proliferation assay, after which cells were exposed to NO donor at various concentrations (0, 5, and 10 μM) for 0, 24 and 48 h. The data are the mean ± S.D. (n = 3)

NO donor (μM)	Relative Cell Proliferation		
	0 h	24 h	48 h
Control	1 ± 0.13	1.32 ± 0.11	1.78 ± 0.09
5	1 ± 0.16	1.45 ± 0.12	1.73 ± 0.06
10	1 ± 0.14	1.37 ± 0.09	1.85 ± 0.09

Table 3. The relative change of migration of H460 cell was evaluated using a wound-healing assay, after which cells were exposed to NO donor at various concentrations (0, 5, and 10 μM) for 7 days and 14 days. The data are the mean ± S.D. (n = 3)

NO donor (μM)	Relative Change of Migration			
	Day 7		Day 14	
	12 h	24 h	12 h	24 h
Control	1.00 ± 0.47	1.00 ± 0.81	1.00 ± 0.34	1.00 ± 0.63
5	1.43 ± 0.66	1.13 ± 0.67	2.30 ± 0.77	1.37 ± 0.31
10	1.73 ± 0.35	1.18 ± 0.93	2.47 ± 0.45	1.61 ± 0.55

Table 4. The relative of p-FAK, total FAK, p-Akt, total Akt, Cdc42, and Cav-1 level was analyzed using Western blotting, after which cells were exposed to NO donor at various concentrations (0, 5, and 10 μM) for 7 days and 14 days. The data are the mean ± S.D. (n = 3).

NO donor (μM) / Protiens	Relative protein level					
	Day 7			Day 14		
	0	5	10	0	5	10
p-FAK	1.00 ± 0.49	1.10 ± 0.53	3.13 ± 0.28	1.00 ± 0.37	2.44 ± 0.23	6.55 ± 0.44
total FAK	1.00 ± 0.54	0.93 ± 0.49	0.88 ± 0.72	1.00 ± 0.83	0.93 ± 0.42	0.90 ± 0.29
p-Akt	1.00 ± 0.46	2.63 ± 0.75	3.17 ± 0.47	1.00 ± 0.64	2.77 ± 0.23	3.41 ± 0.35
total Akt	1.00 ± 0.30	0.88 ± 0.49	1.00 ± 0.21	1.00 ± 0.45	1.08 ± 0.61	1.09 ± 0.72
Cav-1	1.00 ± 0.43	3.99 ± 0.21	4.24 ± 0.64	1.00 ± 0.42	3.82 ± 0.74	4.65 ± 0.31
Cdc42	1.00 ± 0.38	2.31 ± 0.33	3.36 ± 0.24	1.00 ± 0.65	2.98 ± 0.19	3.91 ± 0.28

Table 5. The expression level of Cav-1 protein in the control H460, H460/Cav-1, and H460/ShCav-1 cells was analyzed using Western blotting. The data are the mean ± S.D. (n = 3).

Cell types	Relative Cav-1 level
H460/control	3.97 ± 0.19
H460/Cav-1	10.32 ± 0.71
H460/shCav-1	1.00 ± 0.23

Table 6. The expression level of phosphorylated FAK, total FAK, phosphorylated Akt, and total Akt in H460/ShCav-1 and H460/Cav-1 cells was analyzed using Western blotting, after which cells were exposed to NO donor at various concentrations (0, 5, and 10 μM) for 14 days. The data are the mean ± S.D. (n = 3).

Protiens	**Relative protein level**					
	H460/shCav-1			**H460/Cav-1**		
	NO donor (μM)			**NO donor (μM)**		
	0	**5**	**10**	**0**	**5**	**10**
p-FAK	1.00 ± 0.32	1.00 ± 0.18	1.00 ± 0.21	1.00 ± 0.26	3.55 ± 0.37	3.73 ± 0.29
total FAK	1.00 ± 0.39	0.95 ± 0.49	0.91 ± 0.20	1.00 ± 0.19	0.93 ± 0.18	0.96 ± 0.38
p-Akt	1.00 ± 0.61	0.98 ± 0.25	1.02 ± 0.54	1.00 ± 0.55	3.47 ± 0.22	3.68 ± 0.14
total Akt	1.00 ± 0.18	1.12 ± 0.44	1.10 ± 0.29	1.00 ± 0.43	1.03 ± 0.83	1.00 ± 0.52

VITA

Miss. Arpasinee Sanuphan was born on March 26, 1985 in Ratchaburi. She received her Bachelor of Pharmacy (B.Pharm.) with 2nd class honor from Faculty of Pharmacy, Chiang Mai University in 2010.