จุฬาลงกรณ์มหาวิทยาลัย

# ทุนวิจัย

# กองทุนรัชดาภิเษกสมโภช

**รายงานผลการวิจัย**

**บทบาทของไนตริกออกไซด์ สารอนุมูลอิสระออกซิเจนและอนุพันธ์**

**ในการควบคุมการแพร่กระจายของเซลล์มะเร็งปอด**

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**ชื่อโครงการวิจัย** บทบาทของไนตริกออกไซด์สารอนุมูลอิสระออกซิเจนและอนุพันธ์ในการควบคุมการแพร่กระจายของเซลล์มะเร็งปอด

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**บทคัดย่อ**

ความสามารถของเซลล์มะเร็งในการดื้อต่อการตายแบบอะนอยคิส(anoikis) การเคลื่อนย้ายและรุกรานไปยังเนื้อเยื่อล้อมรอบนั้นเกี่ยวข้องกับศักยภาพในการแพร่กระจายระดับสูงและระยะลุกลามของมะเร็งเมื่อไม่นานมานี้พบว่าโปรตีนคาวีโอลิน-1 (caveolin-1) ได้รับความสนใจเพิ่มมากขึ้นโดยเฉพาะบทบาทที่เกี่ยวข้องกับพฤติกรรมที่รุนแรงของเซลล์มะเร็งผู้วิจัยพบว่าไนตริกออกไซด์ (nitric oxide) และไฮโดรเจนเปอร์ออกไซด์ (hydrogen peroxide) มีบทบาทสำคัญในการยับยั้งกระบวนการอะนอยคิสของเซลล์มะเร็งปอดผ่านกลไกที่ขึ้นกับคาวีโอลิน-1โดยหน้าที่ของคาวีโอลิน-1ในการยับยั้งอะนอยคิสผ่านทางกลไกที่เกี่ยวข้องกับ Mcl-1การศึกษานี้พบว่าคาวีโอลิน-1ควบคุม Mcl-1 ผ่านปฏิกิริยาระหว่างโปรตีนและยับยั้งการนำสัญญาณในเซลล์อะนอยคิสการศึกษาทาง immunoprecipitation และ immunocytochemistry แสดงให้เห็นว่าคาวีโอลิน-1 เกิดปฏิกิริยากับ Mcl-1 และป้องกัน Mcl-1 จากการทำลายผ่านทางวิถี ubiquitin-proteasome ซึ่ง Mcl-1 และกลุ่มของ Mcl-1-Cav-1 สูงขึ้นในเซลล์ที่มีการแสดงออกเพิ่มขึ้นของคาวีโอลิน-1 แต่กลับลดลงอย่างมากในเซลล์ที่ลดการแสดงออกของคาวีโอลิน-1ผลการศึกษานี้สอดคล้องกับการที่ Mcl-1 ubiquitination ลดลงอย่างมีนัยสำคัญเมื่อเพิ่มการแสดงออกของคาวีโอลิน-1 และเพิ่มขึ้นเมื่อลดการแสดงออกของคาวีโอลิน-1นอกจากนี้ผู้วิจัยพบว่าคาวีโอลิน-1มีบทบาทสำคัญในการเคลื่อนย้ายและรุกรานของเซลล์ H460 ซึ่งเป็นเซลล์มะเร็งปอดในมนุษย์และผลกระทบนี้ถูกควบคุมอย่างแตกต่างกันโดยอนุมูลอิสระในเซลล์ซุปเปอร์ออกไซด์แอนไอออน (superoxide anion) และไฮโดรเจนเปอร์ออกไซด์ลดการแสดงออกของคาวีโอลิน-1 และยับยั้งการเคลื่อนย้ายและรุกรานของเซลล์ในขณะที่ไฮดรอกซิลเรดิคาล (hydroxyl radical) เพิ่มการแสดงออกของคาวีโอลิน-1และสนับสนุนการเคลื่อนย้ายและรุกรานของเซลล์ผลการยับยั้งของซุปเปอร์ออกไซด์แอนไอออนและไฮโดรเจนเปอร์ออกไซด์ต่อคาวีโอลิน-1เกี่ยวพันกับกลไกที่ไม่ขึ้นกับการถอดรหัสโดยเกี่ยวข้องกับการทำลายโปรตีนผ่านวิถี ubiquitin-proteasome

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|---|---|
| **Project Title** | Roles of nitric oxide, reactive oxygen species, and their derivatives in regulation of lung cancer cell metastasis |
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## Abstract

The capability of cancer cells to resist to anoikis, migrate and invade surrounding tissues is associated with high metastatic potential and advanced stage of cancers. Recently, caveolin-1 (Cav-1) protein has garnered increased attention in implicating the aggressive behavior of cancer cells. We demonstrate herein that nitric oxide and hydrogen peroxide play a role in inhibiting anoikis process of lung cancer cells via caveolin-1 dependent mechanism. The Cav-1 function in inhibition of anoikis was demonstrated to be cause through Mcl-1 dependent mechanism. The present study demonstrated that Cav-1 regulates Mcl-1 through protein-protein interaction and inhibits its down-regulation during cell anoikis. Immunoprecipitation and immunocytochemistry studies showed that Cav-1 interacted with Mcl-1 and prevented it from degradation via the ubiquitin-proteasome pathway. Mcl-1 and Mcl-1-Cav-1 complex were highly elevated in Cav-1-overexpressing cells but were greatly reduced in Cav-1 knockdown cells. Consistent with this finding, we found that Mcl-1 ubiquitination was significantly attenuated by Cav-1 overexpression but increased by Cav-1 knockdown. Furthermore, we revealed herein that Cav-1 plays an important role in the migration and invasion of human lung carcinoma H460 cells and that these effects are differentially regulated by cellular ROS. Superoxide anion and hydrogen peroxide down-regulated Cav-1 expression and inhibited cell migration and invasion, whereas hydroxyl radical up-regulated the Cav-1 expression and promoted cell migration and invasion. The down-regulating effect of superoxide anion and hydrogen peroxide on Cav-1 is mediated through a transcription-independent mechanism that involves protein degradation via the ubiquitin-proteasome pathway.

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# List of Abbreviations

| Abbreviations or symbol | Term |
|---|---|
| CAT | catalase |
| Cav-1 | caveolin-1 |
| DMNQ | 2, 3-dimethoxy-1, 4-naphthoquinone |
| $FeSO_4$ | Ferrous sulfate |
| HCav-1 | pEX_Cav-1 plasmid transfectants H460 |
| $H_2$DCF-DA | 2′, 7′-dichlorodihydrofluorescein diacetate |
| HMcl-1 | pCDNA3.1-hMcl-1plasmid transfectants H460 |
| $H_2O_2$ | hydrogen peroxide |
| LAC | Lactacystin |
| Mcl-1 | myeloid cell leukemia-1 |
| MnTBAP | Mn(III)tetrakis(4-benzoic acid)porphyrin chloride |
| Mock | pcDNA3 control plasmid transfectants H460 |
| MTT | 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide |
| NaFM | sodium formate |
| NSCLCs | Non small cell lung cancer cells |
| PI | propidium iodide |
| ROS | reactive oxygen species |
| shCav-1 | shRNA-Cav-1 plasmid transfectants H460 |
| XTT | 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide |

# Introduction

According to the widely accepted concept, the cancer microenvironment is critical in the facilitation of metastasis (Rofstad, 2000 and Isaiah, 2002) and causes significant impacts on cancer cell behaviors such as anoikis resistance, chemotherapeutic resistance, invasion, and migration (Rennebeck et al., 2005).

Among various mediators found in cancer microenvironments, nitric oxide (NO) and reactive oxygen species (ROS) have garnered most attention in cancer research. Elevated NO and increased ROS have been associated in many human lung cancers (Liu et al., 1998; Arias-Díaz et al., 1994; Fujimoto et al., 1997; Yang et al., 1998 and Thompson, 1998). However, the role of nitric oxide and its mechanism of metastasis regulation in association with anoikis resistance are still unclear. Nitric oxide has been reported to have both pro- and anti-apoptotic effect on cells, depending on a variety of factors, including cell type, cellular redox status, and the flux and dose of local NO (Heigold et al., 2002; Iyer et al., 2008). In human lung carcinoma cells, we previously reported that NO plays a suppressive role in apoptosis induced by a variety of agents, including Fas death ligand (Chanvorachote et al., 2005), chemotherapeutic agent cisplatin (Chanvorachote et al., 2006), and the metal carcinogen chromium (Azad et al., 2006). However, the role of NO in cell anoikis and its potential regulation of Cav-1 have not been well investigated.

Regarding ROS, increased oxidative stress and ROS production have also been associated with many human metastatic tumors, including the lung (Misthos et al., 2005 and Chung-man Ho et al., 2001), breast (Brown and Bicknell, 2001), prostate (Lim et al., 2005), colon (Szatrowski and Nathan, 1991), andovary (Hileman et al., 2004). ROS such as superoxide anion, hydrogen peroxide ($H_2O_2$), and hydroxyl radical have been shown to

be up-regulated in the tumor microenvironment and have been suggested to play a role in tumor progression and metastasis (Laurent et al., 2005 and Storz, 2005). Although ROS are likely to affect cancer cell motility and invasiveness through multiple mechanisms, Cav-1 may play a key role in this process because Cav-1 is known to be abnormally regulated in invasive tumors and to play a role in cancer cell migration (Williams et al., 2005; Ho et al., 2002; Yoo et al., 2003; Moon et al., 2005 and Cassoni et al., 2009). Cav-1 is also subject to regulation by ROS (Volonte et al., 2002 and Esme et al., 2008), but the underlying mechanism of regulation and the specific ROS involved are unclear.

Resistance to anoikis, a form of apoptotic cell death induced by loss of cell anchorage to extracellular matrixes, has been accepted as a key determinant of cancer cell metastasis (Boisvert et al., 2009 and Chiarugi and Giannoni, 2008). Recently, a number of proteins have been identified to facilitate anoikis resistance in various cancer types. Among these, Caveolin-1 (Cav-1) has perhaps received the most attention since its expression has been linked to cancer progression and aggressiveness (Thompson et al., 2010). Although, some evidence have suggested the tumor suppressing role of Cav-1 (Engelman et al., 1998; Hurlstone et al., 1999 and Racine et al., 1999), in lung cancer Cav-1 potentiates cancer progression and aggressiveness. Cav-1 expression has been shown to relate to poor prognosis and reduced tumor-free periods in lung cancer patients (Ho et al., 2007). Moreover, Cav-1 was shown to facilitate metastasis and induce anoikis resistance in lung carcinoma cell lines (Chanvorachote et al., 2009; Rungtabnapa et al., 2011 and Yeh et al., 2009). Not only does Cav-1 play a role in cell death and survival, but also in cell migration (Luanpitpong et al., 2010), invasion (26), and lipid transportation (Quest et al., 2004). Cav-1 was reported to exhibit scaffold function and to be essential in regulating several proteins such as endothelial nitric oxide synthase (eNOS), G protein

subunit, and non receptor tyrosine kinases (Lajoie and Nabi, 2010), supporting the wide range of activities of this protein in various cellular processes.

The pro-survival member of Bcl-2 family protein named myeloid cell leukemia sequence 1 (Mcl-1) has recently been implicated as a key regulator of cell anoikis (Simpson et al., 2008). In melanoma, the depletion of Mcl-1 renders mutant B-RAF melanoma cells sensitive to anoikis (Boisvert et al., 2009). Likewise, Mcl-1 degradation and Bim up-regulation are a critical determinant of anoikis initiation in wild-type and c-Src-transformed NIH3T3 fibroblast cells. This protein is degraded through the ubiquitin-proteasomal pathway after cell detachment (Woods et al., 2007). Increasing evidence also indicates the role of Mcl-1 in progressive prostate cancer (Zhang et al., 2010), supporting its clinical significance in cancer metastasis.

The objective of the study is to investigate the possible relationship between Cav-1 and Mcl-1 and their regulation of anoikis in lung cancer cells. The hypothesis of this study is that Cav-1 mediates its effect on cancer cell anoikis through Mcl-1 regulation. Using gene overexpression and knockdown strategies, we demonstrated this relationship and elucidated the important role of Cav-1 in regulating Mcl-1 through protein interaction and stabilization, thus revealing the existence of a novel mechanism of anoikis regulation which could be important in cancer metastasis. Metastasis is a multistep process composed of cancer cell detachment, migration, extravasation, and adhesion of the detached cells to other target sites. A key mechanism in the regulation of metastasis is anoikis or detachment-induced apoptosis.

Previous studies have shown that Cav-1 acts as a negative regulator of anoikis (Luanpitpong et al., 2010 and Maurer et al., 2006), and its elevated expression in lung carcinoma is closely associated with the increased metastasis capacity and poor survival of the patients (Michels et al., 2005).

# Survey of Related Literature

## Lung cancer and metastasis

Lung cancer is the leading cause of cancer death in the United States and throughout the world. Non-small-cell lung cancer (NSCLC), one category of lung cancer, is accounted for 80% of overall lung cancer that had been diagnosed. From 1995 to 1999, cigarette smoking and exposure to secondhand smoke accounted for approximately 440,000 annual deaths in the United States. Worldwide, approximately 4 million people die annually from tobacco-attributable diseases, and this number is predicted to rise to 8.4 million by 2020 [Rosell et al., 2004].

### 1.1 Classification of lung cancer

Base on histological type, lung cancer can be classified into different subgroup which is necessary for clinical management and prognosis of the disease. The majority of lung cancers are carcinomas, malignancies that arise from epithelial cells. The two most prevalent histological types of lung carcinoma, categorized by the size and appearance of the malignant cells scored by a histopathologist under a microscope: non-small cell and small-cell lung carcinoma [Travis et al., 1995]. The non-small cell type is the most prevalent type among various lung cancer cells.

Non-small cell lung cancer (NSCLC) frequently presents as advanced, metastatic disease that is primarily treated with systemic chemotherapy. In addition, many patients with earlier stage, potentially curable, NSCLC relapse at distant metastatic sites, resulting in the dismal 5-year survival rate of only 15% for all patients with this disease [Wilson et al., 2009]. Primary NSCLC themselves most commonly metastasize to the adrenal glands, pancreas, liver, brain, and bone [Wilson et al., 2009; Vaporciyan 2000]. These highlight

the metastatic potential of NSCLC which turns from curable to the difficult to treat disease.

### 1.2 Cancer metastasis

During the development of most cancers, rapid cell growth generated some malignant strain that have their abilities to dissociate from their primary tumor, invade adjacent tissue and survive in the blood circulation or lymphatic system that allow them to travel to distant site and settle at new location and then form new colonies. This phenomenon can be termed as metastasis, which is the cause of 90% of human cancer deaths. [Hanahan and Weinberg 2000].

Metastasis is exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. As a barrier to developing metastases, cells normally undergo apoptosis after they lose contact from neighbouring cells or from their extra cellular matrix (ECM). This cell death process has been termed ‘‘anoikis” [Fig. 1] which is necessary to maintain normal cell growth. Tumor cells that acquire malignant potential have developed mechanisms to resist anoikis and thereby survive after detachment from their primary site and while travelling through the lymphatic and circulatory systems [Craig et al., 2008].

#### Anoikis and mechanisms of anoikis resistance

As anoikis resistant capability is the key determinant that enable cancer cells to succeed their colonization at the secondary site, intensive investigate to explore the mechanisms of anoikis resistance have been took place but still remain elusive and may dependent on particular context. The change in protein expression especially the marker of epithelial to mesenchymal transition (EMT) is one mechanism to explain how the cancer cells become

anoikis resistance [Nieman et al., 1999]. Another explanation dealing with the integrins which sense the mechanical forces between cells and ECM, however, when the cell detached from their ECM, the unligated integrins will act as a cell death promoters through the integrin-mediated death (IMD) process [Stupack et al., 2001]. However, integrin signaling can be ignored and the cells can bypass the survival signal that obtained from constitutively activate downstream pro-survival signals, such as PI3K, Ras-Erk, NF-kB and Rho GTPase [Tsuji et al., 2009]. These resulted from autocrine secretion of growth factors, such as basic fibroblast (bFGF), hepatocyte growth factor (HGF) [Li et al., 2003].

In clinical study of NSCLC, two main types of protein that frequently found overexpressed in this type of tumor were Caveolin-1 (Cav-1) and Myeloid cell leukemia -1 (Mcl-1) protein [Ho et al., 2007; Song and Coppola, 2005]. However, the anoikis resistance mechanism regarding with Cav-1 and Mcl-1 in NSCLC is still far from clear.In general, the mechanism (s) involved in making cancer cells anoikis-resistant may comprise the stimulation of alternative (i.e. non-ECM) survival signals and/or the inhibition of apoptotic pathways.

**Caveolin-1**

**Definition and function**

Caveolin-1, a 22-kDa scaffold protein, is the essential constituent of caveolae, flask-shaped (50–100 nm) invaginations that can occupy up to20% of the plasma membrane (Fig. 2). Caveolin-1 belongs to a highly conserved gene family and is co-expressed with caveolin-2 in cells and tissues of various origins including mesenchymal, endo/epithelial, neuronal/glial. The caveolin-1 gene is composed of three exons and alternatively translated into the endoplasmatic reticulum (ER) as a full-length 178 amino acids α-isoform and a β-isoform lacking the first 32 aa [Fig. 2]. Structural topology of caveolin-1

is membrane spanning, where both N-termini and C-termini are exposed to the cytoplasm. A central membrane spanning domain (TMD), C- and N-terminal membrane attachment domains (MAD) and three palmitoyl groups at the C-terminus enable its insertion into the inner leaflet of the membrane. Caveolin-1 binds to cholesterol and sphingolipids within ‘‘lipid rafts” which are considered as specialized ‘‘detergent-insoluble cholesterol- and glycolipid-rich” (DIG) membrane microdomains.

When cav-1 completely translated and moved out from endoplasmic reticulum as an monomers, they assemble into higher molecular weight to become homo or hetero oligomers by using their oligomerization domain (amino acid 61-101), resulting in the forming of striated caveolar coat structure named caveolae. This particular structure plays an important role in regulating versatile cellular transport processes such as cholesterol efflux, clathrin-independent endocytosis, lipid and proteFstupin sorting [Sanna et al., 2007]. Moreover, Cav-1 was found in various vesicle-like compartment inside the cell including caveosome, exocytic vesicle, cytoplasmic/lipid-droplet associated form. Cav-1 also reported to localize at nuclear localization by chromatin-immunoprecipitations assay, however, this finding still needs further validation [Williams and Lisanti, 2005]

**Caveolin-1 is an interacting molecule**

Domain that exerts the interaction between cav-1 and another partner molecules is caveolin-1 scaffolding domain (CSD) (residues 82-101). Interestingly, only a select group of peptides showed high affinity binding to the CSD; several lines of studies showed that the prefer sequences matched the following motifs: ΦXΦXXXXΦ, ΦXXXXΦXXΦ, ΦXΦXXXXΦXXΦ, where Φ is an aromatic residue (Phe, Tyr or Trp). Furthermore, a study of known cav-1 interacting molecules demonstrated that at least one such motif could be found in their sequence, indicating that these regions are particularly important for direct interaction with the cav-1 [Couet et al., 1997]. Such interactions are mostly

inhibit the interacting protein function if that interaction occurs at the active catalytic domain, the example of this inhibition effect from direct interaction of cav-1 is practical significance in the case of tyrosine and serine kinases.

**Caveolin-1 and tumorigenesis**

Several lines of evidence including the *in vivo* studies have been shown that Cav-1 expression may attribute to the aggressiveness of cancer. Evidences regarding the role of Cav-1 in promoting drug resistance were demonstrated in human lung carcinoma, ovarian caricinoma, colon adenocarcinoma and breast adenocarcinoma cell lines, all of these drug resistant variants were found overexpression of Cav-1 [Lavie et al., 1998]. Morover, highly invasive phenotype of lung adenocarcinoma had elevated Cav-1 levels [Ho et al., 2002]. Strikingly, secreting Cav-1 from prostate cancer cells were taken up by tumor cells and endothelial cells may promote tumor angiogenesis [Tahir et al., 2001]. Studies in lung metastases of mice with prostate cancer were found to have increased Cav-1 expression compared with the primary tumor. In human, the lymph node metastases of human prostate and breast cancers were shown to have higher Cav-1 level than those of normal epithelial tissues from prostate and breast [Yang et al., 1998]. These in vivo studies highlighted the role of Cav-1 as an oncogenic, premetastatic potential. Indeed, several types of cancer in human patients having increased in Cav-1 level were found decreasing in survival rate. However, contradictory results have also been obtained from mice with breast cancer, genetic deletion of Cav-1 in this animal model induces the increasing in tumorigenesis and lung metastasis. To reconcile some of these contradictory finding, some the model also proposes the Cav-1 levels vary during the course of tumor progression. In early stage, the down regulation of Cav-1 is necessary for facilitating oncogenic transformation, however, in some tumor, the reexpression of Cav-1 at later stage possibly confer to its potential to become drug resistance and metastatic cancer.

**Caveolin-1 and anoikis resistance**

In the metastatic potential point of view, the ability of cancer cells to become metastasized is may depended on an extent of anoikis resistance. Current evidences demonstrated that Cav-1 seems to regulate anoikis resistance in various *in vitro* studies. The studies performed in breast cancer MCF-7, the overexpression of Cav-1 induces the cells to become fully resistant to anoikis by inhibiting the activation of p53 which then further suppressed $p21^{WAF1/Cip1}$ [Ravid et al., 2006].

In attached condition, integrin engagement normally provide survival signal through phosphatidyl inositol-3-kinase (PI3K)/Akt signaling by sensing the mechanical forces arising from the extracellular matrix (ECM). Integrins are a family of αβ-heterodimeric cell-surface receptors that mediate attachment to the extracellular matrix. Integrins associate with signaling molecules in the focal adhesion complex, which serves as a signaling device and provides a direct link to the cytoskeleton [Rungtabnapa et al., 2011]. As a consequence, when the cells undergo detachment from primary site, the loss of integrin engagement will further reduce survival signal through PI3K/Akt and culminate in apoptosis.

It is well documented that cav-1 can activate PI3K/Akt pathway which promote the cell survival. Cav-1 has been shown to interact with and inhibit serine/threonine protein phosphatases PP1 and PP2A, leading to sustained Akt activation and inhibition of apoptosis by thapsigargin in prostate cancer cells [Kozopas et al., 1993]. In addition, a transient increase in the activity of the tyrosine kinases c-Src and c-Fyn after the detachment of intestinal epithelial cells was shown to be mediated by PI3K pathway and Cav-1. This transient activation of Src-family kinases (SFK) plays crucial role in the transient protection against anoikis in these cell [Loza-Coll et al.,2005]. Taken together,

cav-1 promotes anoikis resistant in cancer cells by providing survival signal through PI3K/Akt pathway when the cells lose their integrin engagement upon detachment.

**Myeloid cell leukemia-1 (Mcl-1)**

**Definition and function**

Mcl-1 (myeloid cell leukemia 1) is a pro-survival member of the Bcl-2 family that was initially identified as an immediate-early gene expressed during PMA-induced differentiation of ML-1 myeloid leukemia cells [Vaux et al., 1988]. Mcl-1 was identified sequence similarity to the previously known pro-survival protein Bcl-2. Bcl-2 family protein promoted oncogenesis regarding its abilities of maintaining cell viability through inhibition of apoptosis [Lutz, 2000]. According to the characteristic of Bcl-2 family proteins that can regulate apoptosis, virtually all malignancies were found to be dysregulated of Bcl-2 family expression. Unlike others anti-apoptotic Bcl-2 family members, Mcl-1 contained 3 putative BH domains resembles to proapoptotic protein Bax. However, its large N-terminal region contained regulatory motif that could participated in its anti-apoptotic function (Fig. 5). Mcl-1 differs from its pro-survival relatives in its larger size of 350 residues, as compared to Bcl-2 at 239 residues (Fig. 4) and Bcl-2 like protein X (Bcl-$X_L$) at 233 residues. Residues 170–300 of Mcl-1 share a great deal of structural and functional homology to both Bcl-2 and Bcl-$X_L$, containing its three BH domains (Bcl-2 and Bcl-X possess 4), which confer the ability to heterodimerize with other family members[Lutz, 2000].The ability of Mcl-1 that can inhibit apoptosis involved its ability to sequester proapoptotic proteins Bcl-1 homologous antagonist killer (Bak) and Bcl-2-associated protein X (Bax) which localized at the mitochondrial membrane and in the cytoplasm, respectively. Both Bak and Bax trigger the release of cytochrome c into the cytoplasm by their abilities to form pores in the mitochondrial membrane. The released cytochrome c induces the activation of a family of

cysteine proteases named caspase which are responsible for the degradation of many macromolecules that were observed during the apoptotic processes. The interaction of Mcl-1 with the subset of BH3-only Bcl-2 family protein can result in different apoptotic outcome, if Mcl-1 interacts with BH3-only pro-apoptotic protein, it will inhibit Bak and Bax polymerization and prevent apoptosis. On the other hands, the Mcl-1 repressing effects on the polymerization of Bak and Bak can be reduced by the degradation of Mcl-1 or the interaction with another subset of BH-3 only protein, which further allow the release of Bak and Bax from Mcl-1 and promotes apoptosis. Because Mcl-1 sometimes found to be localized at mitochondrial membrane, the ability of Mcl-1 to be localized at the membrane could be due to its transmembrane domain at the C-terminal portion of Mcl-1, the deletion of this region inhibits membrane insertion and localization of the protein [Akgul et al., 2000].

**Mcl-1 and tumorigenesis**

Since Mcl-1 was identified as an early response gene induced during the differentiation of ML-1 human myeloblastic leukaemia cells, it draws first attention on its role in haematological malignancies. In the *in vivo* model, transgenic mice expressing Mcl-1 under the control of the endogenous Mcl-1 promotor show enhanced survival of B and T cells which finally developed myeloid malignancy [Zhou et al., 2001]. Antisense experiments have demonstrated that the expression of Mcl-1 is required for the multiple myeloma cells survival [Zhang et al., 2002]. Likewise, in the clinical treatment of plasma cell malignancy, a *mcl-1* antisense therapeutic strategy has been more advocacy than the *bcl-2* antisense [Derenne et al., 2002]. However, the role of Mcl-1 in cancer tumorigenesis is not specific to only haematological malignancies, in various kind of solid tumors were found the elevated level of Mcl-1 and its expression also impact on the cancer cell aggressiveness. In hepatocellular carcinoma (HCC), the ectopic expression of

miR-101 significantly inhibited the ability of hepatoma cells to form colonies *in vitro* and to develop tumors in nude mice. This miR-101 was found that it may exert its pro-apoptotic function in HCC by targeting Mcl-1 which was shown by the reduction of Mcl-1 endogenous protein level and repressed the expression of luciferase carrying the 3'-untranslated region of Mcl-1 [Su et al., 2009]. In another cancer type, Mcl-1 was strikingly linked with poor prognosis of human breast cancer, in which the high level of Mcl-1 was related to high tumor grade and poor survival of breast cancer patients. Moreover, in various kinds of cancer cell line including breast cancer, Mcl-1 stabilization by GSK-3β inactivation that is involved with the phosphorylation at $Ser^{9}$ allows Mcl-1 to be more pronounce effects in tumorigenesis in that such cancer [Ding et al., 2007].

**Mcl-1 and anoikis resistance**

Because Mcl-1 is the member of the Bcl-2 family protein, the role of Mcl-1 in anoikis regulation is relied heavily on the mitochondrial pathway. Upon cell detachment, the loss of signal from PI3K/Akt enable Bim releasing from dynein complex and then translocate to the mitochondria to support Bax, Bak oligomerization which then culminate in triggering cytochrome c release and caspase activation [Akiyama et al., 2009]. However, in the present of sufficient amount of Mcl-1, it will sequester Bim at the mitochondrial membrane, thereby preventing activation of related BH3-only factors that function to induce apoptosis [Opferman et al., 2003]. Researchers found a tightly controlled balance between Mcl-1 and Bim, as Mcl-1 reduction was insufficient to stimulate Bax in the absence of Bim expression [Woods et al., 2007). Additionally, inhibition of Mcl-1 reduction and over-expression of Bim had opposite effects on cell survival; the first resulted in anoikis resistance, while the second induced a dose-dependent cell death response.

# Procedure

## Cells and reagents

Non small cell lung cancer (NSCLC)-H460 cells and melanoma G361 cells were obtained from American Type Culture Collection (Manassas, VA). H460 cells were cultured in RPMI 1640 medium, while G361 cells were cultured in DMEM medium. RPMI 1640 was supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/ml penicillin/ streptomycin. DMEM was supplemented with 10% FBS, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. All cell cultures were incubated in a 5% $CO_2$ environment at 37°C. Lactacystin (LAC), MG 132, dimethysulfoxide (DMSO) were obtained from Sigma Chemical, Inc. (St. Louis, MO); propidium iodide (PI) and Hoechst 33342 from Molecular Probes, Inc. (Eugene, OR); rabbit Cav-1 antibody, rabbit Mcl-1 antibody, mouse monoclonal ubiquitin antibody, mouse monoclonal Cav-1 antibody and peroxidase-conjugated secondary antibody from Abcam (Cambridge, MA); MitoTracker Red CMXRos, Alexa Fluor 350 goat anti-mouse IgG (H+L), Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Antibody for ubiquitin, protein G-agarose bead, and β-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

## Plasmid and transfection

The Cav-1 expression plasmid pEX_Cav-1 was obtained from American Type Culture Collection (Manassas, VA),the Mcl-1 expression plasmid 25375:pCDNA3.1-hMcl-1was obtained from Addgene (Cambridge, MA), and Cav-1 knockdown plasmid shRNA-Cav-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Stable transfection of cells with Cav-1 expression plasmid or Cav-1 knockdown plasmid was performed by culturing H460 cells in a 6-well plate until they reached approximately 60% confluence.

15 μl of Lipofectamine reagent and 2 μg of Cav-1, shRNA-Cav-1 or mock pcDNA3 control plasmid were used to transfect the cells in the absence of serum. After 12 h, the medium was replaced with culture medium containing 5% FBS. Approximately 36 h after the beginning of transfection, the cells were digested with 0.03% trypsin, and the cell suspensions were plated onto 75-ml culture flasks and cultured for 24 to 28 days with G418 selection (600 μg/ml). The stable transfectants were pooled and the expression of Cav-1 protein in the transfectants was determined by Western blotting. The cells were cultured in antibiotic-free RPMI 1640 medium for at least two passages before used in each experiment.

**Anoikis assay**

For anoikis evaluation, 6-well tissue culture plates were coated with 200 μl (6 mg/ml in 95% ethanol) of poly 2-hydroxyethylmethacrylate (poly-HEMA; Sigma) and left for 10 h in a laminar flow hood. Cells in a single cell suspension were seeded in poly-HEMA-coated plates at the density of $1\times10^5$ cells/ml and incubated for various times up to 24 h at 37°C. Cells were harvested, washed, and incubated with 20 μM of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) for 4 h at 37°C. Optical density was then determined using V-max photometer (Molecular Devices, Menlo Park, CA) at the wavelength of 450 nm. Absorbance ratio of treated to non-treated cells was calculated and presented as relative cell viability. For Hoechst 33342 and PI assays, cells were incubated with 10 μM of Hoechst 33342 or 15 μM of PI for 30 min at 37°C. Apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were scored under a fluorescence microscope (Olympus IX51 with DP70).

**Soft agar colony formation assay**

The anchorage-independent cell growth was determined by assaying colony formation in soft agar. Briefly, monolayer cells were prepared into a single-cell suspension by trypsinization and homogenization. Cells were suspended in culture medium and 0.33% low melting temperature agarose, and then 2 ml containing 2 x $10^4$ cells were seeded in a 35mm dish over a 3 ml layer of solidified culture medium with 0.6% agarose. The medium was refreshed every three days. The colonies were attained by light microscope and photographed after 2 weeks of incubation at 37˚C.

**Western blotting**

After specific treatments, cells were incubated in lysis buffer containing 20 mMTris-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 a commercial protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland) for 30 min on ice. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Equal amount of proteins of each sample (40 μg) were denatured by heating at 95°C for 5 min with Laemmli loading buffer, and subsequently loaded on 10% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 hour in 5% nonfat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween-20) and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase–coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced with chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL) and quantified using analyst/PC densitometry software (Bio-Rad).

**Immunoprecipitation**

Cells are washed after treatment and lysed in lysis buffer at 4°C for 20 min. After centrifugation at 14,000 g for 15 min at 4°C, the supernatants were collected and determined for protein content. Cell lysates were normalized and equal amount of protein per sample (60 μg) were incubated with anti-Cav-1 antibody conjugated to protein G plus-agarose beads (Santa Cruz) for 6 h at 4°C. The immune complexes were washed five times with ice-cold lysis buffer, resuspended in 2x Laemmli sample buffer, and boiled at 95°C for 5 min. Immune complexes were separated by 10% SDS-PAGE and detected for Cav-1 and Mcl-1 complexes by Mcl-1 antibody. For detecting ubiquitin-Mcl-1 complex, the anti-Mcl-1 antibody was incubated with the cell lysate in the immunoprecipitation step followed by Western blot analysis using anti-ubiquitin antibody.

**Quantitative real time RT-PCR**

One microgram of Trizol-extracted RNA was reverse-transcribed in a 100 μl reaction mixture containing 500 μM dNTP, 125 units of Multi Scribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), 40 units of RNase inhibitor, 2.5 μM oligo (dT), 1x Taq Man reverse transcriptase buffer, and 5 mM $MgCl_2$ at 48°C for 40 min. The primer for *Mcl-1* (Hs03043899_m1*) and 18s rRNA (Hs99999901_s1) were obtained from Applied Biosystems. Amplification was performed at the following cycling conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A SYBR Green PCR Master Mix (Applied Biosystems) was used with 1 ng of cDNA and with 100-400 nM primers. A negative control without any cDNA template was run with every assay. All PCR reactions were performed by using ABI PRISM7900 Sequence Detection System (Applied Biosystems). Relative mRNA levels were determined by using the comparative $C_T$ (threshold cycle) method, where the Mcl-1 target

is normalized to the control and compared with a reference sample (assigned a relative value of 1) by the equation: $2^{-\Delta\Delta CT}$.

**Immunofluorescence**

Cells ($0.5\times10^6$/well) were seeded in 6-well plates for 24 h to allow the cell to completely adhere to the surface. Then, the cells were fixed in 3.7% formaldehyde for 10 min at room temperature, and then permeabilized and blocked in a solution containing 0.5% saponin, 1% FBS, and 1.5% goat serum for 30 min. After primary antibody incubation with both Cav-1 mouse monoclonal antibody (Abcam) at 1:100 dilution and Mcl-1 rabbit polyclonal antibody (Abcam) at 1:100 dilution for 1 h, cells were washed and incubated together with Alexa Fluor 350 goat anti-mouse IgG (H+L) conjugated secondary antibody (Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (H+L) conjugated secondary antibody (Invitrogen) for 30 min. Mitochondria were stained with MitoTracker Red CMXRos (Invitrogen). Cells were cytospun onto a glass slide and mounted using the anti-fade reagent Fluoromont-G (Southern Biotech, Birmingham, AL). Images were acquired by confocal laser scanning microscopy (Zeiss LSM 510).

**ROS detection**

Intracellular ROS were determined using dichlorofluorescein-diacetate (DCF $H_2$-DA) as a specific ROS probe. After specific treatments, cells were incubated with 10 μM of DCF $H_2$-DA for 30 min at 37°C, after which they were washed, trypsinized, resuspended in phosphate-buffered saline (PBS), and immediately visualized for fluorescence intensity by fluorescence microscope.

**Apoptosis/anoikis and necrosis evaluation**

After treatment for indicated times, cells were added with 10 μg/mL of Hoechst 33342 and/or 5 μg/mL of propidium iodide (PI), followed by visualized under fluorescence microscope (Olympus IX51 with DP70). The number of anoikis / apoptosis cell was

calculated to represent the percentage of apoptosis/ anoikis cells in each sample. The use of different dyes for mode of cell death determination was based on the dissimilar characteristic between necrotic and apoptotic / anoikis cells. Cells that were undergoing apoptosis / anoikis demonstrate cell shrinkage, chromatin condensation, membrane blebbing, DNA fragmentation and apoptotic body formation, by which cell membrane was not broken up in early apoptosis / anoikis. Whereas necrosis cell death was resulted in loss of membrane integrity, swelling and disruptive of the cells. Hoechst 33342 was a permeable dye, which has ability to stain nucleic acid of apoptotic / anoikis and necrosis cells. Whereas PI was an impermeable dye staining necrosis cells that have lost membrane integrity and show red PI staining throughout the nuclei. The apoptotic / anoikis and necrotic cells can be thus distinguished under fluorescence microscope.

Apoptosis was also assayed by cell cycle analysis. Cells were centrifuged, washed with PBS and resuspended in propidium iodide 50 μg/ml with 0.1% Triton X-100 for 20 min at 4°C. Cells were analyzed by flow cytometry and the sub G0/G1 fraction was used as a measure of the apoptotic percentage.

**Statistical analysis**

Mean data from independent experiments were normalized to result in cells in the control. All the experiments were repeated at least three times. A statistical analysis between two groups was verified by Student's *t* test, in comparison to multiple groups, an analysis of variant (ANOVA) with post hoc test was conducted. The strength of relationships, correlation coefficient (*r*), between each protein level after detachment was determined with SPSS version 16 (SPSS Inc., Chicago, IL, USA). A *P*-value of less than 0.05 would be considered as statistically significant.

# Results

## 1. Effect of nitric oxide and reactive oxygen species on lung cancer cells anoikis and caveolin-1 expression

### Nitric Oxide Inhibits Anoikis in H460 lung cancer cells

Anoikis was induced by detaching the cells and incubating them in attachment-resistant poly-HEMA-coated plates for various times and analyzed for cell viability by XTT assay. Fig.1A shows that detachment of the cells caused a time-dependent decrease in cell viability with approximately 55 and 15% of the cells remaining viable after 6 and 12 h, respectively. Analysis of cell apoptosis by flow cytometry using FITC-labeled annexin V antibody shows a significant increase in annexin V-associated cellular fluorescence as early as 6 h after the detachment and reached a maximum at about 18 h (Fig. 1B). In contrast, analysis of cell necrosis using PI as a probe shows no significant increase in the PI signal over a 24-h period. These results suggest that apoptosis is the primary mode of cell death after detachment of H460cells. Morphologic analysis of apoptotic cell death by fluorescence microscopy using Hoechst 33342 and annexin V-FITC further confirms the results (Fig. 1E). To investigate the role of NO in detachment-induced apoptosis, detached H460 cells were treated with various concentrations of NO donors and inhibitors, and their effect on cell survival was determined by XTT assay. Fig. 1Cshows that treatment of the cells with NO donor, SNP, or DETANONOate caused a dose-dependent decrease in cell death, whereas treatment of the cells with NO inhibitor, AG, or PTIO had an opposite effect. Analysis of cell apoptosis by annexin V-FITC and Hoechst 33342 assays similarly shows the inhibitory and promoting effect of the NO donors and inhibitors, respectively, on detachment-induced cell death (Fig. 1, D and E). The NO

donors and inhibitors, when used at the indicated concentrations, had no significant effect on cell necrosis as determined by PI assay (Fig. 1D).

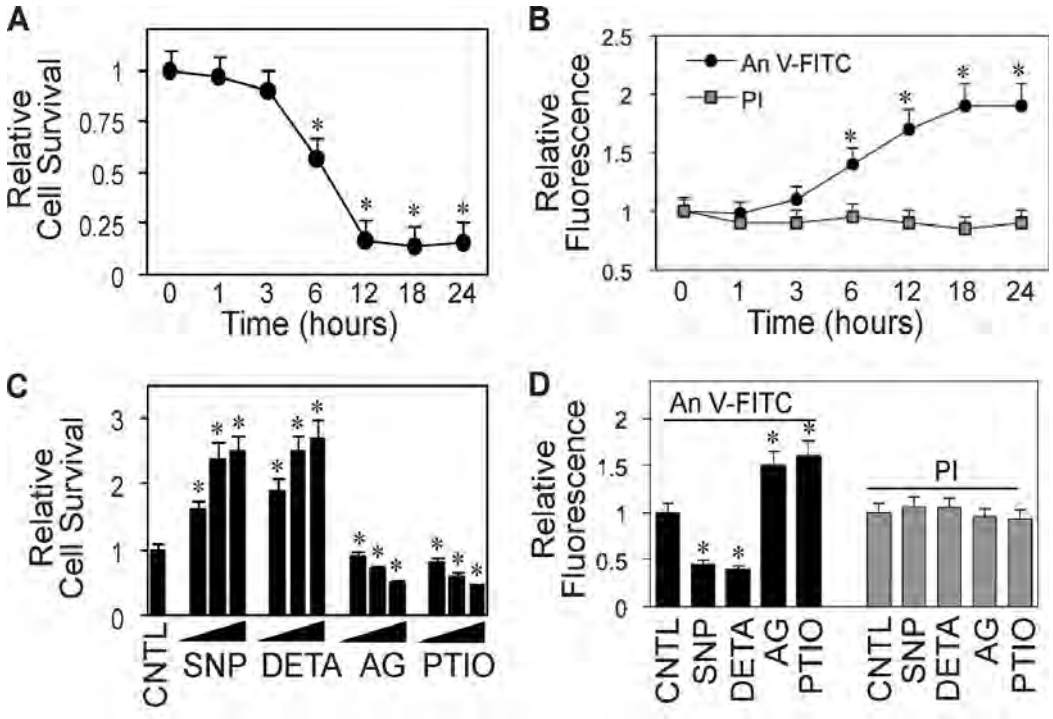


Figure 1.Detachment-induced apoptosis and its inhibition by NO.A, effect of cell detachment on cell survival determined by XTT assay. Lung epithelial H460 cells were detached and suspended in HEMA-coated plates for various times (0–24 h). B, effect of cell detachment on apoptosis and necrosis determined by flow cytometry using annexin V-FITC (An V-FITC) and PI assays. C, effect of NO modulators on detachment-induced cell death. Detached cells were treated with various concentrations of NO donor, SNP (10, 50, 100 μM), or DETA NONOate (10, 50, 100 _M) or with NO inhibitor, AG (100, 200, 300 μM), or PTIO (10, 50, 100 μM) for 12 h. Cell survival was then determined by XTT assay. CNTL, control.D, effectsof NO modulators on detachment-induced apoptosis

and necrosis. Detached cells were treated with SNP (50 μM), DETA NONOate (50 μM), AG (300 μM), or PTIO (50 μM) for 12 h, and cell apoptosis and necrosis were determined as described above. Data are the mean ± S.D.(n = 3). *, $p< 0.05$ versus non-treated control.

**Nitric Oxide Prevents Detachment-induced Cav-1 Down-regulation**

We further investigated the potential regulation of Cav-1 by NO. Cells were detached and suspended in HEMA coated plates in the presence or absence of NO donors and inhibitors. Cav-1 protein expression was then determined by Western blotting. Fig. 2shows that the NO donors SNP and DETA NONOate strongly inhibited detachment-induced Cav-1 down-regulation. In contrast, the NO inhibitors AG and PTIO promoted this down-regulation (Fig. 2). These results were confirmed by confocal immunofluorescence microscopy which shows that Cav-1 fluorescence intensity was reduced after cell detachment and that the NO donor SNP was able to inhibit this reduction, whereas the NO inhibitor AG further reduced the signal intensity (Fig. 2).

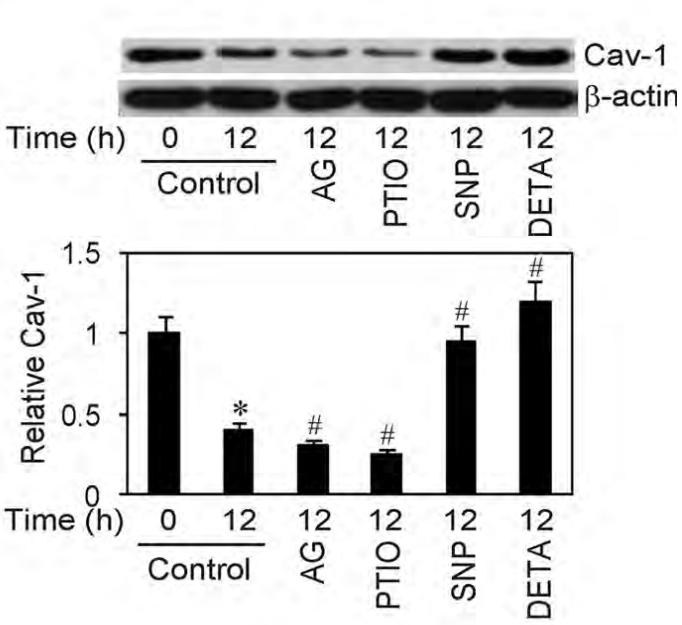


Figure 2.Effect of cell detachment on Cav-1 expression and its regulation by NO. Detached cells were treated with NO inhibitor, AG(300 μM) or PTIO (50 μM), or with NO donor, SNP (50 μM), or DETA NONOate (50 μM) for 12 h, after which they were analyzed for Cav-1 expression by Western blotting. Data are the mean ± S.D.(n = 3). *, $p< 0.05$ versus non-treated control.

**Effect of reactive oxygen species on cell anoikis.**

We determined the role of specific ROS in the regulation of cell anoikis. Detached cells were treated with specific ROS scavengers as earlier described, and cell viability was determined by XTT assay. Figure 3Ashows that treatment of the cells with Catalase (CAT) or NAC decreased cell viability as compared with non-treated control, whereas treatment with MnTBAP or NaF had no significant effect. These results indicate that hydrogen peroxide ($H_2O_2$) is the primary oxidative species regulating anoikis of H460 cells after detachment.

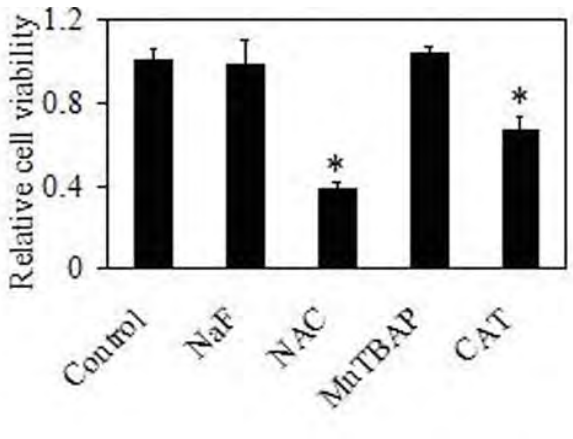


Figure 3.Effect of reactive oxygen species scavengers on cell anoikis. H460 cells were detached and left untreated or treated with the specific ROS scavengers NaF (5 mM), NAC (10 mM), MnTBAP (100 μM), and CAT (1,000 U/ml). Viability of detached H460 cells was determined by XTT assay. Data are the mean ± S.D.(n = 3). *, $p< 0.05$ versus non-treated control.

**Effect of reactive oxygen species on caveolin-1 expression.**

We tested whether ROS regulate anoikis through Cav-1. H460 were detached and suspended in poly-HEMA-coated plates in the presence or absence of specific ROS scavengers, and Cav-1expression was determined by Western blotting. Figure 4 shows that CAT and NAC were able to decrease Cav-1expression in the cells, whereas NaF and MnTBAP had minimal effect. These results indicate the role of $H_2O_2$as a key regulator of Cav-1 expression which presents a key mechanism of anoikis regulation after cell detachment.

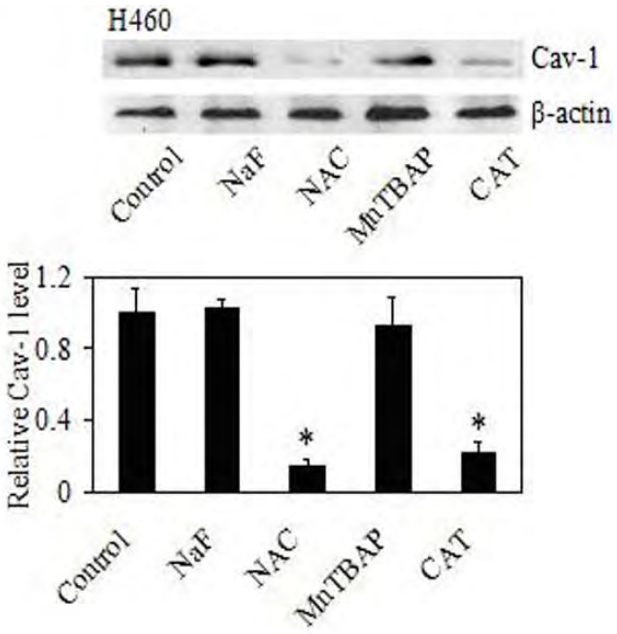


Figure 4. Effect of reactive oxygen species scavengers on Cav-1. H460 cells were detached and left untreated or treated with the specific ROS scavengers NaF (5 mM), NAC (10 mM), MnTBAP (100 μM), and CAT (1,000 U/ml). Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, transferred, and probed with Cav-1 antibody. Data are the mean ± S.D.(n = 3). *, $p< 0.05$ versus non-treated control.

**2. Effect of reactive oxygen species on cancer cells migration**

**Differential effect of reactive oxygen species on cell migration**

ROS have been shown to be involved in several cellular migratory processes, including wound repair, metastasis, and angiogenesis. However, the roles of specific ROS and regulatory mechanisms are not well understood. We tested whether ROS play a role in the migration of lung carcinoma H460 cells and determined the specific ROS involved. Cells were treated with various known inducers and scavengers of ROS, and their effect on cell migration was determined by wound migration assay. Fig. 5 shows that treatment of the cells with MnTBAP, a superoxide dismutase mimetic and scavenger of superoxide anion,

stimulated the migration of cells across the wound space, whereas treatment of the cells with DMNQ, a known inducer of superoxide anion, had an opposite effect. Likewise, treatment of the cells with catalase (CAT) ($H_2O_2$ scavenger) promoted cell migration, whereas treatment with $H_2O_2$ inhibited the migration. These results indicate the inhibitory role of superoxide anion and $H_2O_2$in the migration of H460 cells during wound healing. In contrast to the above findings, treatment of the cells with sodium formate (NaFM), a known hydroxyl radical scavenger, inhibited the migration, although the hydroxyl radical generator ferrous sulfate($FeSO_4$) promoted this effect. These results suggest the differential roles of ROS in the regulation of cancer cell migration.

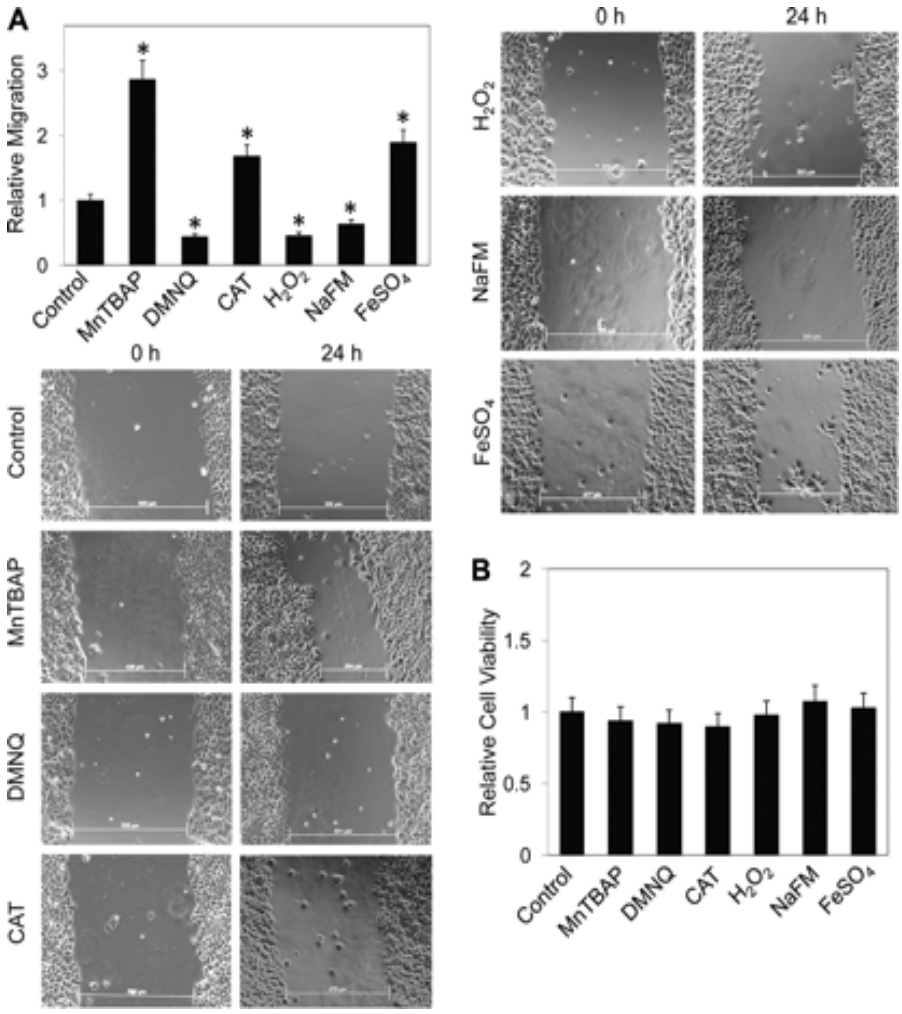

Figure 5. ROS regulate migration of human lung epithelial H460 cells. A, confluent monolayers of H460 cells were wounded, and the cells were allowed to migrate for 24 h in the presence or absence of various ROS modulators, including MnTBAP (50 μM), DMNQ (5 μM), CAT (7,500 units/ml),($H_2O_2$100 μM), NaFM (5 mM), and $FeSO_4$ (50 μM). Wound space was visualized under a phase contrast microscope and analyzed by comparing the relative change in wound space of the treated over nontreated cell monolayers. Representative micrographs from four independent experiments are shown. B, Effect of ROS modulators on cell viability. Cell monolayers were similarly treated with the indicated concentrations of ROS modulators and analyzed for cell viability after 24 h by MTT assay. Data are the mean ± S.D. (n = 3). *, $p< 0.05$ versus non-treated control.

**ROS Regulate Cav-1 Expression**

Cav-1 may be regulated by ROS, which may represent a key mechanism of cell migratory regulation by ROS. To test this possibility, cells were treated with various ROS modulators, and their effect on Cav-1 expression was determined by Western blotting. Fig. 6A shows that treatment of the cells with DMNQ or $H_2O_2$ substantially down-regulated the expression of Cav-1, whereas treatment with $FeSO_4$ up-regulated the expression. Consistent with the inhibitory role of superoxide anion and $H_2O_2$, the scavengers of these ROS (MnTBAP and CAT, respectively) promoted the Cav-1 expression (Fig. 6B). In contrast, the hydroxyl radicals scavenger NaFM inhibited the expression, supporting the positive regulatory role of hydroxyl radicals in Cav-1 expression. This latter result was confirmed by the observation that deferoxamine, a known metal chelator and inhibitor of hydroxyl radicals, also inhibited Cav-1 expression (Fig. 6B).

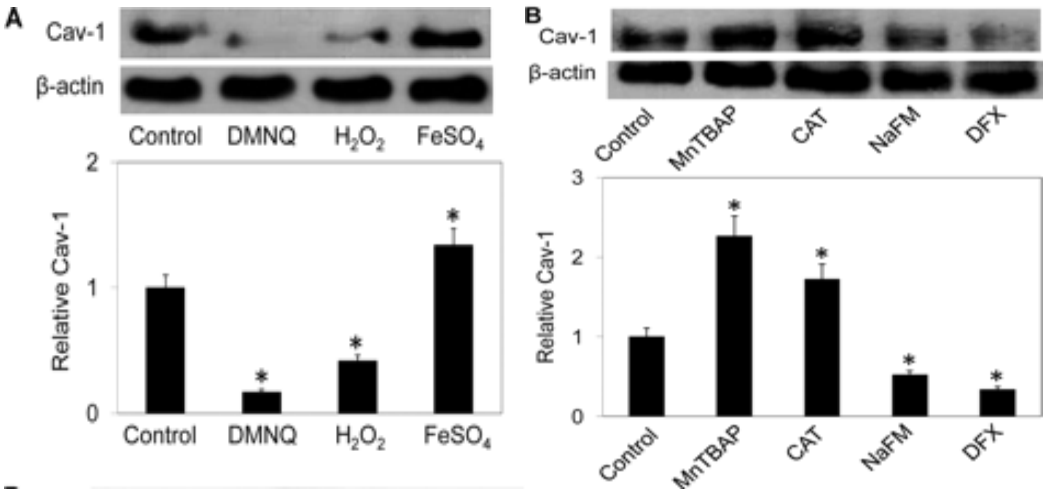


Figure 6.ROS regulate Cav-1 expression. A, H460 cells were treated with various ROS generators for 24 h, and cell lysates were prepared and analyzed for Cav-1 expression by Western blotting. B, cells were treated with various ROS scavengers or inhibitors and Cav-1 expression was determined after 24 h. Data are the mean ± S.D. (n = 3). *, $p< 0.05$ versus non-treated control.

**Role of Specific ROS in Cell Migration, Invasion, and Cav-1Expression**

To determine further the relationship between Cav-1 expression and cell motility regulation by different ROS, cells were treated with various concentrations of ROS modulators, and their effects on cell migration, invasion, and Cav-1 expression were determined. Fig. 7, A and D, shows that DMNQ caused a dose-dependent and parallel decrease in both cell migration and Cav-1 expression. Addition of MnTBAP reversed both of these effects of DMNQ, indicating the role of superoxide anion in the processes and their association. Similar to DMNQ, $H_2O_2$ caused a dose-dependent and concomitant decrease in cell migration (Fig. 7B) and Cav-1 expression (Fig. 7E), both of which were inhibited by catalase. In contrast to superoxide anion and $H_2O_2$, hydroxyl radical promoted cell migration (Fig. 7C) and Cav-1 expression (Fig. 7F) as indicated by their

positive responses to $FeSO_4$ treatment and their inhibition by NaFM co-treatment, which were shown to induce and inhibit, respectively, the formation of hydroxyl radicals. Together, these results indicate the positive regulatory role of hydroxyl radical and the opposing role of superoxide anion and $H_2O_2$ in cell migration through Cav-1 expression. A similar finding was observed with regard to the role of different ROS in cell invasion (Fig. 7G).

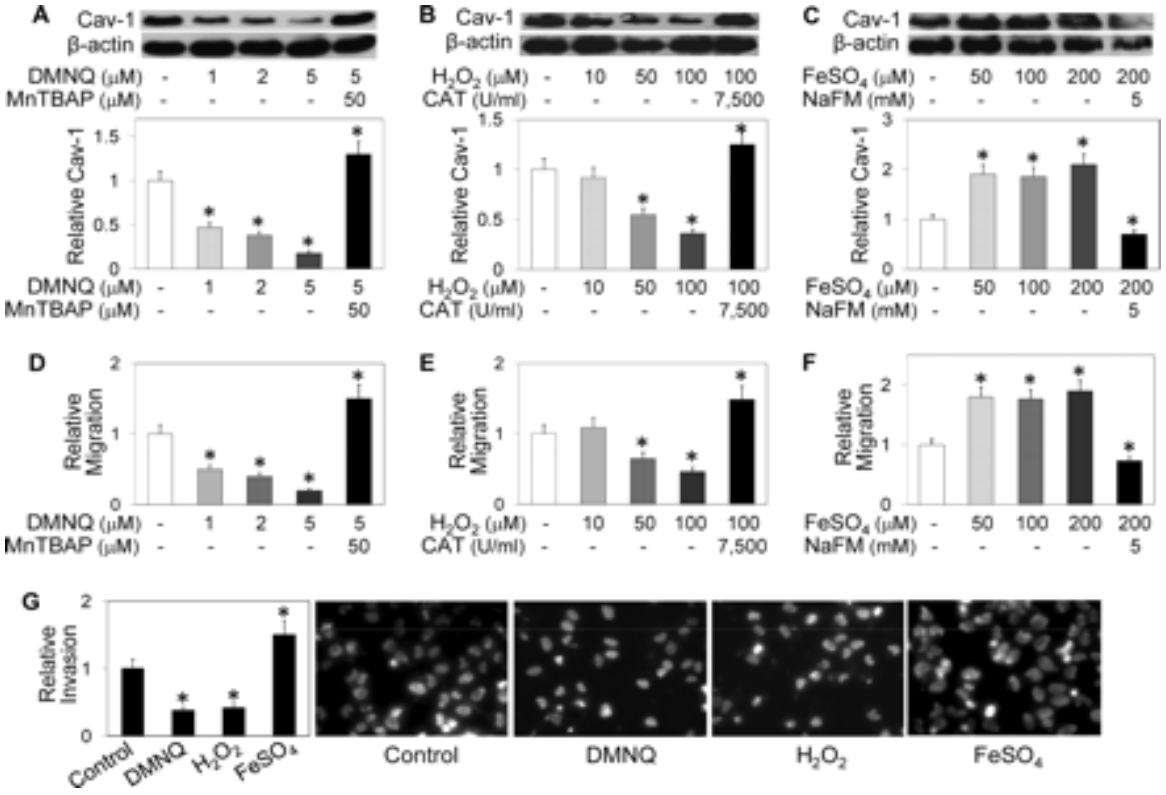


Figure 7.Differential effects of ROS on Cav-1 expression, cell migration, and invasion. Western blot analysis is shown of Cav-1 expression in H460 cells treated with the indicated concentrations of DMNQ and MnTBAP (*A*), $H_2O_2$ and CAT (*B*), and $FeSO_4$ and NaFM (*C*) for 24 h. Cell migration was determined 24 h after the treatment with DMNQ and MnTBAP(*D*), $H_2O_2$ and CAT (*E*), and $FeSO_4$ and NaFM (*F*). Cell invasion was determined 24 h after the treatment with DMNQ (5 μM), $H_2O_2$ (100 μM), and $FeSO_4$ (50 μM) (*G*). Data are the mean ± S.D. ($n$ = 3). *, $p$< 0.05 *versus* nontreated control.

**Cav-1 Promotes Cell Migration and Invasion**

We tested whether Cav-1 can regulate the migration and invasion of lung carcinoma H460 cells. The cells were stably transfected with Cav-1 or control plasmid, and their effects on Cav-1 expression, cell migration, and invasion were determined. Western blot analysis of Cav-1 expression shows a substantial increase in the expression in Cav-1-transfected cells as compared with vector-transfected control (Fig. 8 A). Wound migration assay shows that the Cav-1-transfected cells exhibited an increase in migratory activity as compared with control-transfected cells (Fig. 8). Transwell invasion assay similarly indicates an increase in cell invasiveness in the Cav-1-overexpressing cells as compared with control-transfected cells (Fig.8 C)

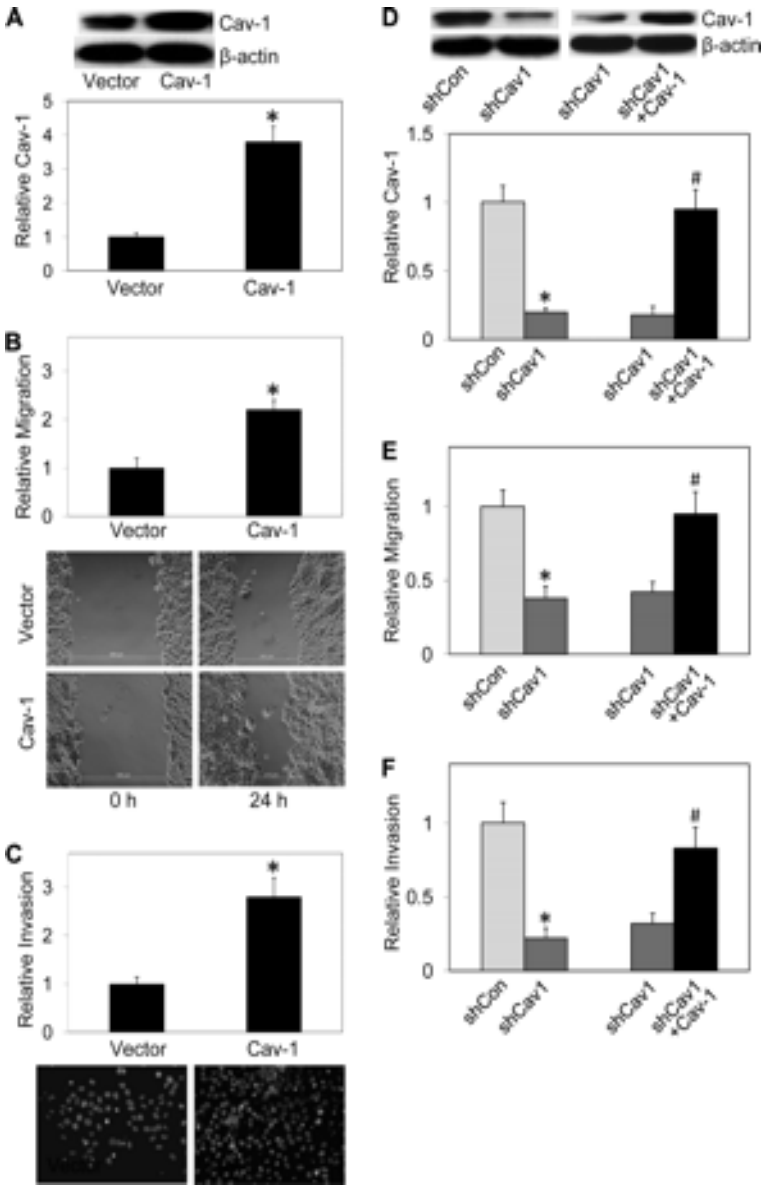


Figure 8.Effects of Cav-1 overexpression and knockdown on cell migration and invasion. H460 cells were stably transfected with Cav-1 or control plasmid. A, Cav-1 expression in the control and Cav-1-transfected cells was determined by Western blotting. Cell extracts were prepared and separated on 10% SDS-polyacrylamide gels, transferred, and probed with Cav-1 antibody. β-Actin was used as a loading control. B, effect of Cav-1 overexpression on cell migration. Cav-1 and control-transfected cells were cultured in 24-well plates and analyzed for cell migration at 24 h by wound assay. C, effect of Cav-1 overexpression on cell invasion. Cav-1 and control-transfected cells were added to

extracellular matrix-coated inserts in a Transwell chamber and incubated for 24 h. Invading cells were counted under a fluorescence microscope after staining with Hoechst 33342, and the average number of cells was scored in each case. D–F, Cav-1 knockdown experiments were performed using H460 cells treated with Cav-1 shRNA (shCav-1) viral particles or control shRNA (shCon) particles as described under "Materials and Methods." D, Cav-1 expression in shCav-1 and shCon-treated cells determined by Western blotting at 36 h post-treatment (left panel). Rescue experiment was performed in shCav-1-treated cells by transfecting the cells with Cav-1 plasmid as described above and analyzed for Cav-1 expression by Western blotting (right panel). E and F, migration and invasion of shCon, shCav-1, and rescued cells determined by wound and Transwell assays, respectively. Data are the mean ± S.D. (n = 3). *, $p < 0.05$ versus control transfection; #, $p < 0.05$ versus shCav1 control.

**Cav-1 Promotes anchorage-independent growth of cells**

We tested whether Cav-1 can regulate the anchorage-independent growth H460 cells. The H460 cells were stably transfected with Cav-1 or shRNA Cav-1 plasmid to increase or knock-down Cav-1, respectively. Western blot analysis of Cav-1 expression showed a substantial increase in Cav-1 protein level in Cav-1-transfected cells, whereas a significant decrease in Cav-1 level was observed in shRNA Cav-1 transfected cells (Figure 9 D). These stable transfected cells then were prepared in suspension and analyzed for anchorage-independent growth by soft agar colony assay. Results indicated that H460/Cav-1 exhibited high capability to grow in the anchorage-independent manner with approximately 2-fold induction of colony diameter and 1-fold induction of number of cell colonies compared to the control vector cells, whereas H460/shCav-1 cells

exhibited low ability to survive and grow (Figure 9 A, B and C). These results indicate that Cav-1 facilitates growth in detached condition.

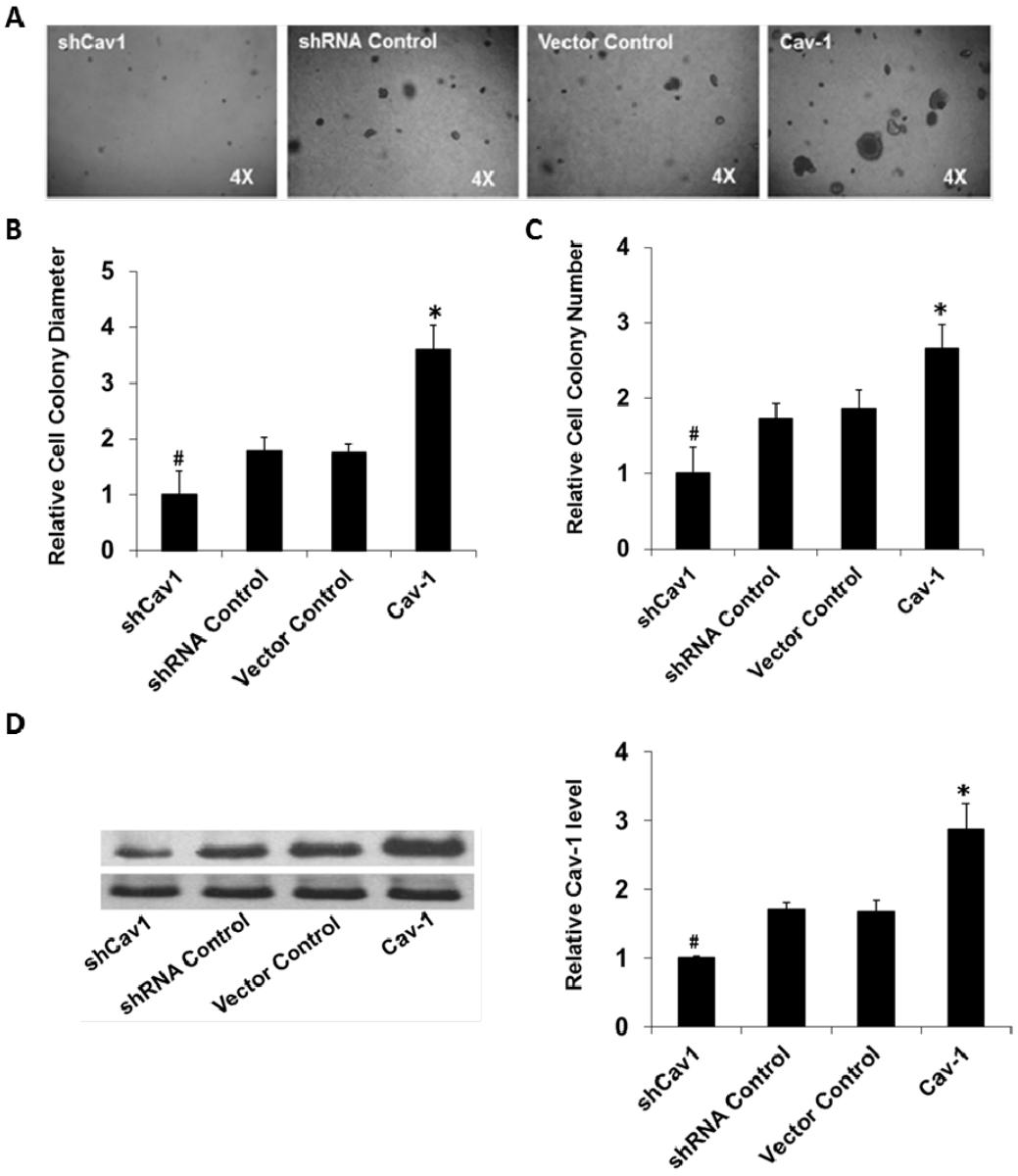


Figure 9.Effects of Cav-1 overexpression and knockdown on anchorage-independent growth of cells. A, H460 cells were stably transfected with Vector, Cav-1 overexpressing, Control shRNA or shRNA Cav-1 plasmids to generate H460/Vector, H460/Cav-1, H460/Control shRNA and H460/shCav1, respectively. The ability of anchorage-independent growth of transfected cells was evaluated by using soft agar colony

formation assay. The relative cell colony diameter was determined by using image analyzer. Values are mean ± SD (n=4). # $p< 0.05$ versus control shRNA; * $p< 0.05$ versus control vector. B, The relative cell colony diameter and C, relative number of colonies were determined by using image analyzer. # $p< 0.05$ versus control shRNA; * $p< 0.05$ versus control vector. D, The Cav-1 protein expression was determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiment were normalized to the control cells.

**3. Role and underlying mechanism of caveolin-1 in regulation of anoikis in lung cancer cells**

**Caveolin-1 inhibits anoikis of H460 cells**

To investigate the role of Cav-1 protein in anoikis regulation of the lung cancer cells, we first characterized the effect of different ectopic Cav-1expressionlevels on cell anoikis of H460 cells. Through stable gene transfection, we generated Cav-1 overexpressing (HCav-1) cells, short-hairpin (sh)RNA knockdown (shCav-1) cells, and vector control (mock) cells. These mutant clones were analyzed for Cav-1 expression by Western blotting (Fig. 10 A). To study anoikis, HCav-1, shCav-1 and vector control cells were detached and incubated in adhesion-resistant poly-HEMA coated plates. Cell survival was then determined at various times by XTT assay. Analysis of cell viability showed that detachment of the cells caused a time-dependent decrease in cell survival with approximately 80%, 50%, and 30% of the HCav-1, mock, and shCav-1 cells respectively remained viable after 6 h (Fig. 10 B). At 24 h post-detachment, HCav-1 cells exhibited ~60% viability, whereas both mock and ShCav-1 cells showed a survival rate of <40%.

Control experiments, in which cells were allowed to attach in normal tissue culture plates, showed no significant change in cell viability over the 24-h test period (data not shown). Analysis of cell apoptosis by Hoechst 33342 assay showed that shCav-1 cells were most susceptible to apoptosis induced by cell detachment, whereas HCav-1 cells were least susceptible (Fig. 10 C). This finding is consistent with the cell viability data showing the highest rate of survival of HCav-1 cells after detachment. Morphological analyses of apoptotic and necrotic cell death by Hoechst 33342 and propidium iodide assays showed that apoptosis was the primary mode of cell death induced by cell detachment in H460 cells (Fig. 10 D).

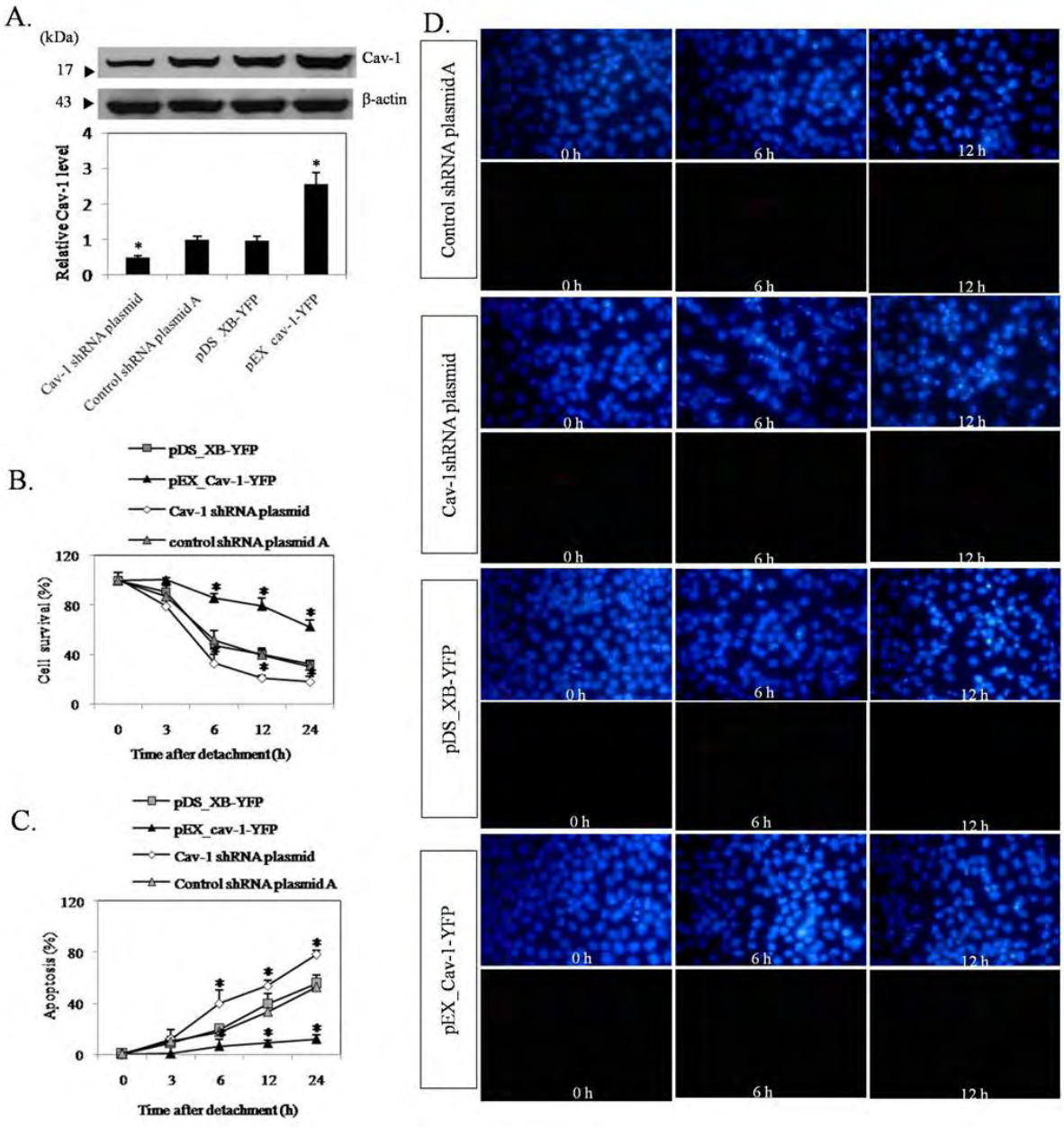


Figure 10. Caveolin-1 overexpression increases anoikis resistance in H460 cells. A, Control transfected (pDS_XB-YFP, control shRNA plasmid A), cav-1 overexpressing cells (pEX_cav-1-YFP) or cav-1 knockdown cells (cav-1 shRNA plasmid) were constructed and grown in culture which then analyzed for Cav-1 expression by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the results. Columns are the mean ± S.D. (n

= 3). *, $P < 0.05$ versus control transfected cells. B, Subconfluent (90%) monolayer of control transfected, cav-1 overexpressing cells and cav-1 knockdown cells were detached and suspended in poly-HEMA coated plates for various times (0-24 h). At the indicated times after detachment, the cells were collected and the survival was determined by XTT assay. Viability of detached cells at time 0 h was considered as 100%. C, Percentage of cell detachment-induced apoptosis was analyzed by Hoechst 33342 nuclear fluorescence. Data point represents the mean ± S.D. (n = 3) *,$P<0.05$ versus control transfected cells. D, Detachment-induced apoptosis and necrosis in control transfected cells, cav-1 overexpressing and cav-1 knockdown cells. Detached cells were suspended in poly-HEMA coated plates for 0-12 h and cell apoptosis and necrosis were determined by Hoechst 33342 and PI fluorescence measurements, respectively.

**Cell detachment induces Cav-1 and Mcl-1 down-regulation**

The role of Cav-1 and Mcl-1 in cancer cell anoikis is unclear. To provide evidence for the role of these proteins, we evaluated the expression profiles of Cav-1 and Mcl-1 after cell detachment in lung cancer H460 cells. The cells were detached, suspended in adhesion-resistant plates, and analyzed for Cav-1 and Mcl-1 protein expression by Western blotting. Figure 11A and B show that after cell detachment, both Cav-1 and Mcl-1 expression gradually decreased over time in concomitant with cell viability and death, suggesting their potential relationship and role in anoikis regulation. Like Cav-1, the role of Mcl-1 in anoikis regulation was studied using stable gene transfection. Figure 11 C shows that stably transfected Mcl-1 (HMcl-1) cells expressed a high level of Mcl-1 protein as compared to vector-transfected control cells. The HMcl-1 cells also showed resistance to anoikis as indicated by their increased viability after cell detachment over control cells (Fig. 11 D).

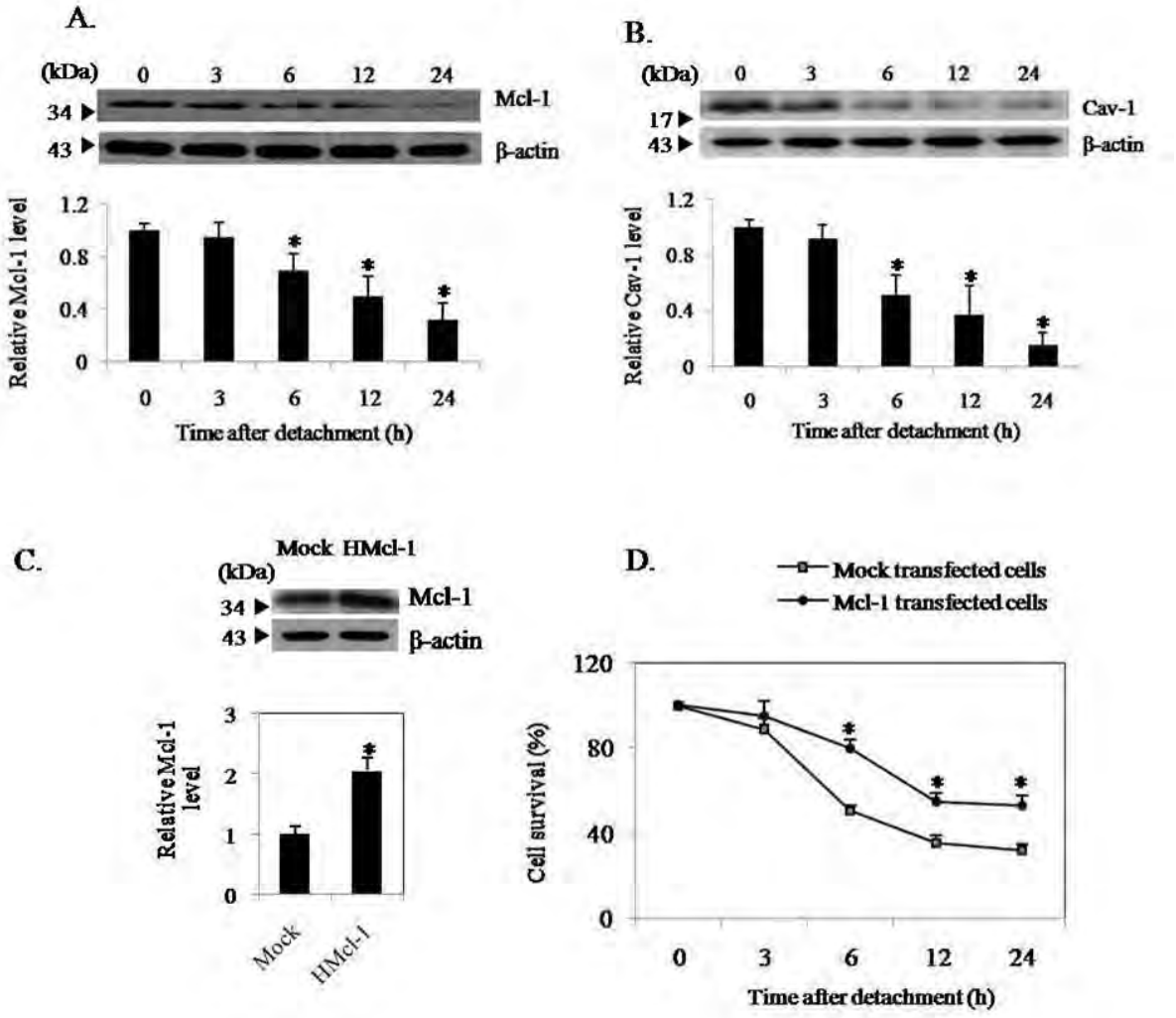


Figure 11.Cav-1 and Mcl-1 expression after cell detachment. A,B H460 cells were detached and suspended in poly-HEMA coated plates for various times (0-24 h). Blots were probed with antibodies specific to Mcl-1 and Cav-1 and were reprobed with β-actin antibody. Columns are mean ± S.D. (n = 3)*, $P < 0.05$ versus control at time 0. C, Mock and HMcl-1 cells were grown in culture and analyzed for Mcl-1 expression by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the results. D, Subconfluent (90%) monolayers of Mock and HMcl-1 cells were detached and suspended in poly-HEMA coated plates for various times (0-24 h). At the indicated times, the cells were collected

and their survival was determined by XTT assay. Viability of detached cells at time 0 was considered as 100%. Data point represent the mean ± S.D. (n = 3) *, $P$ <0.05 versus control transfected cells. Columns are the mean ± S.D. (n = 3). *, $P < 0.05$ versus control transfected cells.

**Mcl-1 downregulation during cell anoikis is regulated by Cav-1 interaction**

Cav-1 has been shown to function as a scaffold protein regulating the stability and function of several proteins. The observations that both Cav-1 and Mcl-1 have a similar effect on anoikis and their expression is similarly downregulated during anoikis suggest the possible linkage and shared mechanism of anoikis regulation. Since Mcl-1 is recognized as a relatively short half-life protein and its scaffolding interaction with Cav-1 has not been reported, we explored their possible interaction by generating a correlation plot between Cav-1 and Mcl-1 expression during cell anoikis (Fig. 12 A). Not only did the reduction of these proteins correlate well with the induction of cell anoikis, but also the plot revealed a highly correlated profile of Cav-1 and Mcl-1 downregulation with the correlation coefficient of 0.98.

Next, we used immunoprecipitation techniques to determine the direct interaction between the two proteins. HCav-1, shCav-1, and mock-transfected cells were detached and suspended in adhesion-resistant plates. Cell lysates were then prepared, immunoprecipitated using Cav-1 antibody, and analyzed for Cav-1-Mcl-1 complex by Western blots usingMcl-1 antibody as a probe. The results showed that Cav-1-Mcl-1 complex formation was most pronounced in HCav-1 cells, which express the highest level of Cav-1, and least expressed in Cav-1 knockdown (shCav-1) cells (Fig. 12 B). These results suggest that Cav-1 plays a role in scaffolding Mcl-1 protein and that its interaction with Mcl-1 may play a role in regulating Mcl-1 level. To provide supporting

evidence for the Cav-1 and Mcl-1 interaction, immunocytochemical studies were performed to evaluate the intracellular localization of the two proteins. Figure 12 C shows immunofluorescent staining of Mcl-1 and Cav-1, which are strikingly similar and supportive of the protein co-localization. Using rhodamine-based MitoTracker probe, we further found that such localization is mainly associated with the mitochondria.

**Cav-1 stabilizes Mcl-1 in H460 cells**

Having shown that Cav-1 interacts with Mcl-1, we further investigated whether such interaction is essential for Mcl-1 stability after cell detachment. Adhered HCav-1, shCav-1, and mock cells were stained with antibodies for Mcl-1, Cav-1, and MitoTracker, and their fluorescent signals were observed by microscopy. While the MitoTracker signals are relatively constant in these cells, the intensities of Cav-1 and Mcl-1 signals in these cells vary greatly (Fig. 12 D). Interestingly, cells that express a high level of Cav-1 (HCav-1) also exhibit a high level of Mcl-1, while those that express a low level of Cav-1 (shCav-1) also show a low level of Mcl-1, suggesting the stabilizing effect of Cav-1 on Mcl-1. To further study this effect, HCav-1, shCav-1, and mock cells were detached and incubated in adhesion-resistant plates for 0-12 h. Western blot analysis of Mcl-1 was then performed at 0, 6, and 12 h post-detachment. Figure 12 E shows that at various times of the detachment, Mcl-1 levels in these cells varied depending on the expression levels of Cav-1 in each cell type. These findings strengthen the above finding that Cav-1 interacts with Mcl-1 and stabilizes the protein under different attachment conditions.

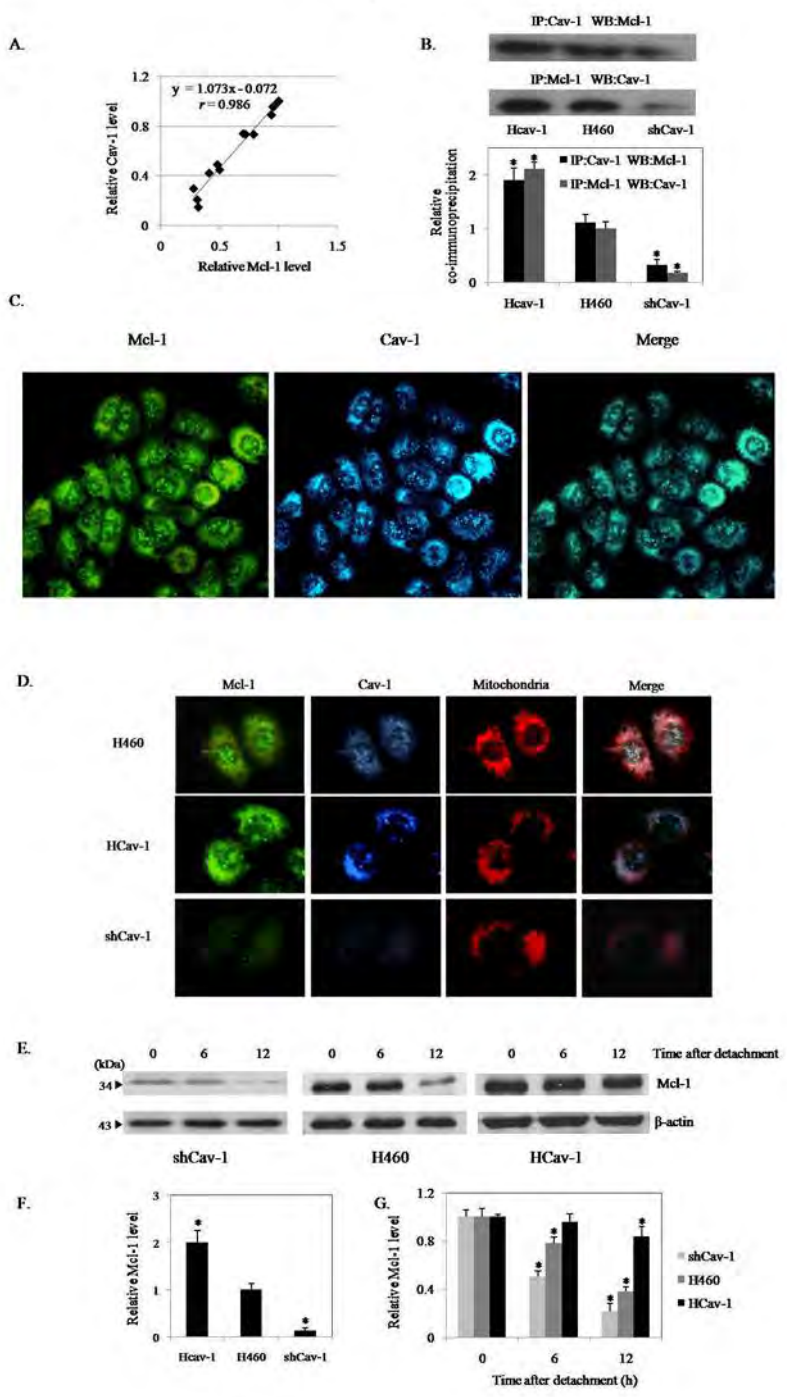


Figure 12.Interaction and localization of Cav-1 and Mcl-1.A, Correlation analysis of the expression of Cav-1 and Mcl-1 after detachment of H460 cells. B, Immunoprecipitation experiments were performed using specific anti-Cav-1 antibody, immunoblots were probed with anti-Mcl-1 antibody and vice versa. Equal amounts of protein (25 μg) were loaded in each lane. Columns are the mean ± S.D (n = 3) *, $P < 0.05$ versus control

transfected cells. C, H460 cells were analyzed for localization of Cav-1 and Mcl-1 by immunofluorescence microscopy. Immunofluorescence was performed using mouse anti-Cav-1 monoclonal antibody and rabbit anti-Mcl-1 polyclonal antibody, followed by appropriate secondary antibodies labeled with Alexa Fluor 350 and Alexa Fluor 488 to visualize Cav-1 and Mcl-1, respectively. Cells were also stained with MitoTracker Red CMXRos (300 nM) to aid visualization of mitochondria. D, Differential expression of Cav-1 and Mcl-1 in HCav-1, shCav-1, and H460 cells. Cells were fixed and processed for immunofluorescence staining. E, Dependence of Mcl-1 reduction after cell detachment on Cav-1 expression. HCav-1, shCav-1, and H460 cells were detached and suspended in poly-HEMA coated plates for various times (0-12 h). Blots were probed with specific antibody to Mcl-1 and were reprobed with β-actin antibody to confirm equal loading of samples. F, Relative Mcl-1 levels in attached cells. G, Relative Mcl-1 levels in shCav-1, HCav-1, and H460 cells after detachment for 0, 6 and 12 h. Columns are the mean ± S.D. (n = 3). *, $P < 0.05$ versus control at detachment time = 0 h.

**Mcl-1 reduction after cell detachment is mediated through ubiquitin-proteasomal degradation**

Although Mcl-1 has been reported to be degraded via the proteasomal pathway, we suspected both transcription and degradation to play a role in Mcl-1 downregulation during cell anoikis. To test this, we performed quantitative real-time RT-PCR and proteasome inhibition studies in detached H460 cells. Mcl-1 mRNA level was significantly reduced as early as 1 hour (data not shown) and remained unchanged up to 24 h after detachment (Fig. 13 A). This finding excluded the possibility that Cav-1 could stabilize Mcl-1 through transcription-dependent mechanism. Therefore, we tested the involvement of ubiquitin-proteasomal system on Mcl-1 downregulation after cell

detachment. Figure 13 B shows that cell detachment caused a substantial reduction in Mcl-1 protein level and that treatment of the cells with specific proteasomal inhibitors, lactacystin and MG132, completely inhibited the Mcl-1 reduction. These results indicate that Mcl-1 downregulation after cell detachment is mediated mainly by the proteasome degradation pathway.

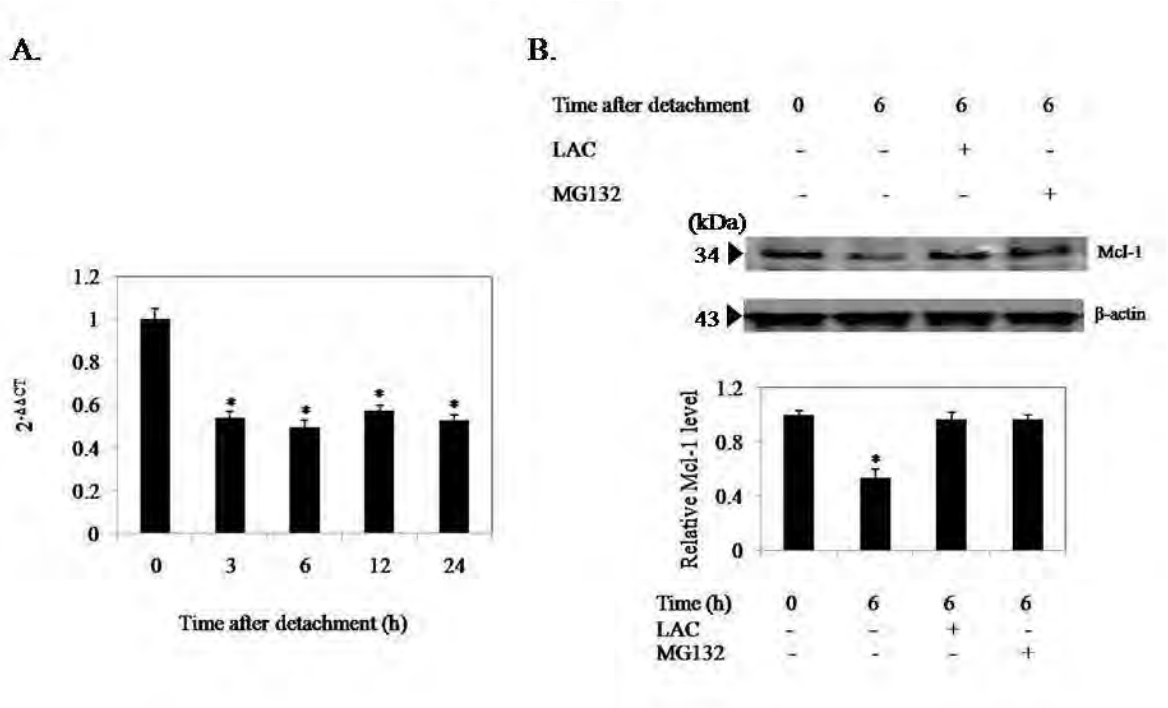


Figure 13.Transcription and degradation of Mcl-1 after cell detachment.A, Real-time PCR analysis of Mcl-1 mRNA expression after cell detachment. The relative mRNA expression was determined by using the comparative $C_T$ method. Columns are the mean ± S.D. (n = 3). *, $P < 0.05$ versus control at detachment time = 0 h. B, Relative Mcl-1 expression after detachment for 0-6 h in the presence or absence of lactacystin (LAC; 20 μM) or MG132 (10 μM) in H460 cells. Columns are the mean ± S.D. (n = 3). *, $P < 0.05$ versus control at detachment time = 0 h.

**Cav-1 stabilizes Mcl-1 by attenuating Mcl-1 ubiquitination**

Proteasomal degradation of a protein is triggered by protein ubiquitination. To test the potential involvement of ubiquitination in Mcl-1 stability and its regulation by Cav-1, Mcl-1 immunoprecipitation and ubiquitination studies were performed in various Cav-1 expressing cells. In normal H460 cells, the formation of ubiquitin-Mcl-1 complexes gradually increased as early as 1 h after cell detachment and peaked at about 6 h (Fig. 14 A). The level of ubiquitin-Mcl-1 complex formation was minimal in Cav-1 overexpressing (HCav-1) cells and maximal in Cav-1 knockdown (shCav-1) cells as compared to vector control (mock) cells (Fig. 14 B). These results indicate that Cav-1 attenuated the ubiquitination of Mcl-1 and stabilized the protein after cell detachment.

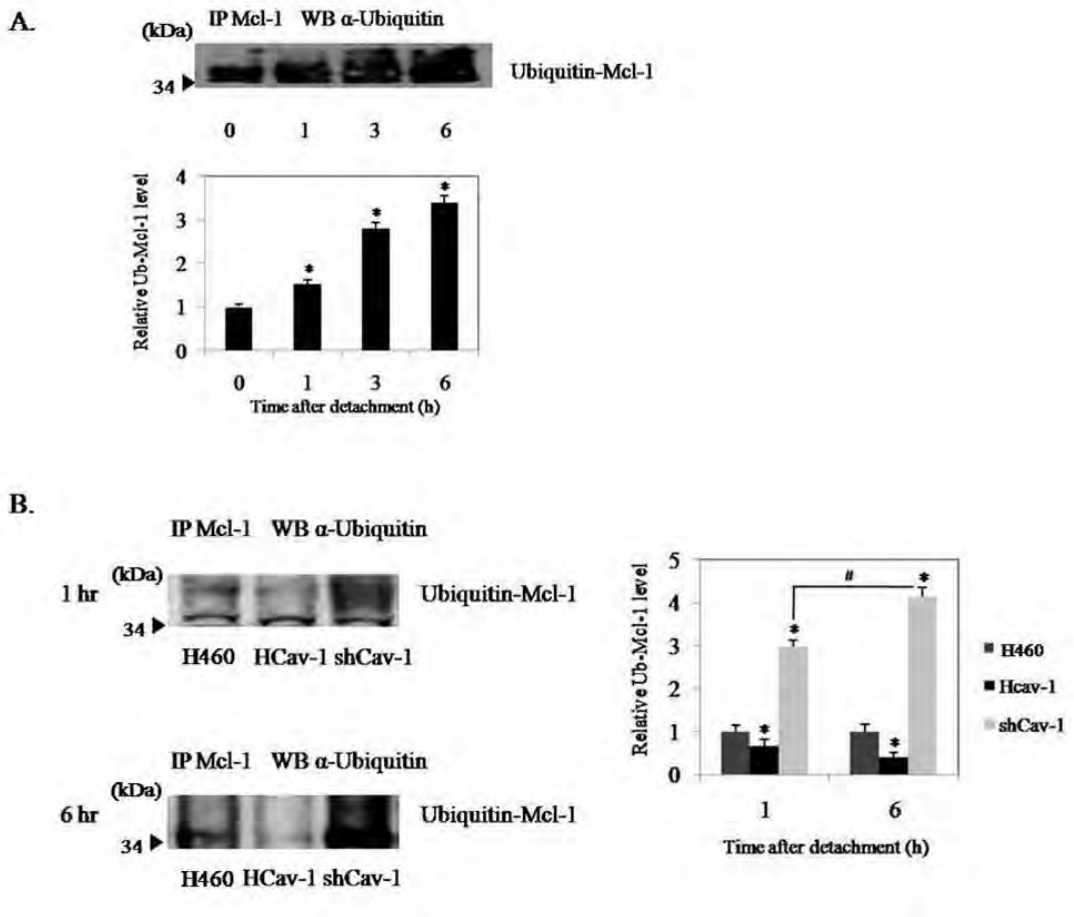


Figure 14.Effect of Cav-1 expression on Mcl-1 ubiquitination. A, H460 cells were detached and suspended in poly-HEMA coated plates for various times. Cell lysates were

prepared and immunoprecipitated (IP) with anti-Mcl-1 antibody. The resulting immune complexes were analyzed for ubiquitin by Western blotting (WB) using anti-ubiquitin antibody. Maximum Mcl-1 ubiquitination was observed at 6 h after cell detachment. The immunoblot signals were quantified by densitometry. Columns are the mean ± S.D. (n = 3). *, $P < 0.05$ versus control at detachment time = 0 h. B, HCav-1, shCav-1, and H460 cells were detached and suspended in poly-HEMA coated plates for 1 and 6 h. Cell lysates were immunoprecipitated (IP) with anti-Mcl-1 antibody and the resulting immune complexes were analyzed for ubiquitin by Western blotting (WB). Columns are the mean ± S.D. (n = 3). *, $P < 0.05$ versus control transfected at detachment time = 1 h; #, $P < 0.05$ versus the indicated control.

## Discussion

Increasing evidence suggests that signaling molecules presenting in the tumor microenvironment have a significant impact on the migratory properties of cancer cells (Kopfstein and Christofori, 2006 and Laurent et al., 2005). For example, increased ROS in the tumor microenvironment has been associated with increased aggressiveness of cancer cells (Dro¨ge, 2002 and Storz, 2005). Although several studies have investigated the effects of ROS on cell migration and invasion, variable results have been reported depending on the type of ROS, dose, and production site, as well as the tissue type of cells (Shim et al., 2007; Kumar et al., 2008; Urbich et al., 2002; Novo et al., 2006 and O'Toole et al., 1996). Several mechanisms of ROS regulation of cancer cell migration have been proposed; most of these involve alterations of cellular cytoskeleton and adhesion molecules. For instance, ROS have been reported to regulate integrin (Chiarugi et al., 2003 and Svineng et al., 2008), small GTPase Rho family proteins (Tobar et al., 2008 and Alexandrova et al., 2006), focal contact-forming proteins (Chiarugi et al., 2003 and BenMahdi et al., 2000), and extracellular matrix-degrading enzymes such as matrix metalloproteinases (Svineng et al., 2008; Nelson and Melendez, 2004 and Lee et al. 2008). So far, the role of specific ROS and their regulatory mechanisms have not been well investigated. We report here that different ROS have different effects on cell migration and invasion in human lung carcinomaH460 cells. Superoxide anion and $H_2O_2$ suppress the migration and invasion of the cells, whereas OH promotes the cell motility activities. Because several ROS are generated during oxidative stress, which has been linked to tumor progression, the results of this study further indicate that depending on the type and abundance of specific ROS generated, oxidative stress conditions may promote or suppress tumor progression by affecting cell migration and invasion. This study also demonstrates the role of Cav-1 as a key target of ROS regulation of cell

motility. Cav-1 has been shown to be involved in cancer cell motility and tumorprogression (Joshi et al., 2008; Han and Zhu, 2010; Fiucci et al., 2002; Sloan et al., 2004 and Williams and Lisanti, 2005). However, its precise role and regulatory mechanisms are still unclear as both promoting and inhibitory roles of Cav-1 have been reported. In pancreatic cancer cells, Cav-1 was shown to inhibit cell migration and invasion through the inactivation of RhoCGTPase and ERK-metalloproteinase signaling pathways (Han and Zhu, 2010 and Lin et al., 2005). A similar inhibitory effect of Cav-1 was observed in breast cancer MTLn3 and MCF-7 cells (Fiucci et al., 2002 and Zhang et al., 2000). In contrast, Cav-1has been reported to promote lung cancer cell invasion by mediating filopodia formation (Ho et al., 2002). Furthermore, Cav-1expression is associated with the tumor grade and metastasis of non-small cell lung cancer (Moon et al., 2005 and Kato et al., 2004). Consistent with the tumor-promoting role of Cav-1, we found that Cav-1promotes the migration and invasion of non-small cell lung cancer H460 cells. The function of Cav-1 is closely associated with its expression level. Although the difference inCav-1 expression may result from various factors such as cell type and stage of cancer (Shatz and Liscovitch, 2008), the tumor microenvironment and oxidative status seem to play a key role. Several ROS have been shown to be up-regulated in the tumor microenvironment and have been implicated in the aggressive behaviors of tumor cells (Laurent et al., 2005 and Storz, 2005). However, the mechanisms by which ROS regulate Cav-1 and tumor cell migration and invasiveness have not been thoroughly explored. In this study, we demonstrate that ROS play an important role in regulating Cav-1 expression and cell migratory functions inhuman lung cancer H460 cells. We also show the positive correlation between Cav-1 expression and cell motility in these cells. More importantly, we demonstrate the differential roles of individual ROS in Cav-1 expression and cell migration with superoxide anion and $H_2O_2$ having a negative regulatory role and

hydroxyl radical playing a positive role. The results of this study also indicate that the effect of ROS on cell migratory functions is dependent on Cav-1 expression and is associated with Akt activity. Activation of Akt by Cav-1has been shown to mediate cancer cell migration (Ravid et al., 2005; Li et al., 2009 and Park and Han, 2009) and is likely to play an important role in the ROS-induced effects on cell motility alterations. ROS may also regulate cell motility through other Cav-1-dependent mechanisms. For example, recent studies have shown that oxidative stress induced Cav-1 phosphorylation at tyrosine 14 (Sun et al., 2009 and Chen et al., 2005), which has been proposed to play a role in cancer cell migration through the regulation of focal adhesion (Joshi et al., 2008).

Regarding nitric oxide, the results of this study also demonstrated that Cav-1 was up-regulated in the cells treated with NO donors. NO has been shown to regulate apoptosis under various physiologic and pathologic conditions (Heigold et al., 2002; Borutaite and Brown, 2003 and Souici et al., 2000); however, its roles in anoikis and metastasis are unclear. Recent evidences suggest that depending on its expression level, NO can exert either promoting or inhibitory effects on tumor growth and metastasis. The promoting effects of NO are generally observed at relatively low but sustained levels of NO, whereas the inhibitory effects of NO are seen at high and acute concentrations that induce tumor cell death (Mocellin et al., 2007; Lala and Chakraborty, 2001 and Monteiro et al., 2004). The results of this study are consistent with previous reports showing that low (micromolar) levels of NO inhibited cell death and increased cell migration (Antonova et al., 2007; Choi et al., 2003 and Dimmeler and Zeiher, 1999). The promoting role of NO in tumorigenesis is also supported by the observations that both inducible and constitutive forms of NO synthase are elevated in several human tumors (Park et al., 2003 and Ambs et al., 1998) and that human and murine carcinomas expressing NO synthase are very aggressive when implanted into mice (Jenkins et al., 1995). Furthermore, there seems to

be a direct correlation between the expression of NO synthase and the tumor grade, suggesting a causative role for NO in promoting metastasis (Jenkins et al., 1995).

When cells are detached from the extracellular matrix, the loss of anchorage-related signals results in an abrogation of certain cellular processes such as cell survival and growth (Hynes, 1999) and consequently initiate the process of anoikis (Frisch and Francis, 1994). Since anoikis is an important cellular event controlling cancer metastasis, unraveling its underlying mechanisms is critical to the understanding of disease pathogenesis and its treatment. Among the many types of cancer, lung cancer has frequently been found to metastasize at the time of tumor detection. While the exact mechanisms of cancer metastasis have been extensively investigated, an upregulation of Mcl-1 (Song and Coppola, 2005) and Cav-1 (Rungtabnapa et al., 2011) has been implicated in lung cancer aggressiveness and progression. Mcl-1 was found to overexpress in NSCLC cells and regulate their survival and sensitivity to diverse apoptotic stimuli (Song and Coppola, 2005). Apoptotic stimuli such as cell detachment induce Bim (activator of BH3-only protein) expression (Cheng et al., 2001). Recently, Zhang *et al*. has demonstrated that Mcl-1 can sequester Bim in NSCLC cells which supports the role of Mcl-1 in attenuating anoikis in this cancer cell type (Zhang et al., 2011). Moreover, amplification or overexpression of Mcl-1 was shown to render cells resistant to detachment-induced apoptosis (Simpson et al., 2008) and the decrease in Mcl-1 level is required in the initiation of cell anoikis (Boisvert et al., 2009 and Woods et al., 2007). Previously, we and others have shown that Cav-1 confers resistance to anoikis in cancer cells (Fiucci et al., 2002 and Rungtabnapa et al., 2011). Furthermore, the expression of Cav-1 has been used as a biomarker for virulence of some cancers (Corn and Thompson, 2010). The role of Cav-1 in cancer cell anoikis has been described in many ways such as the induction of survival pathways (Li et al., 2003) and the reduction

of Cav-1 and Mcl-1 (Rungtabnapa et al., 2011 and Woods et al., 2007). We further demonstrated in this study that during cell anoikis, Cav-1 and Mcl-1 reduction was tightly correlated. Cav-1 functioned as a scaffold protein for Mcl-1 binding as demonstrated by immunoprecipitation studies. In addition, the Cav-1-Mcl-1 complex significantly increased in the Cav-1 overexpressing (HCav-1) cells, but decreased in the Cav-1 knockdown (shCav-1) cells. Immunocytochemistry studies further confirmed the co-localization of Cav-1 and Mcl-1 in the cells which was largely associated with the mitochondria.

Since Mcl-1 is known to be a short half-life protein due to continuous proteasomal degradation (Woods et al., 2007), it is possible that its interaction with Cav-1 could affect its stability which was first demonstrated in this study. Although a rapid decline in Mcl-1 mRNA level was observed at 1 h post-detachment, the mRNA level remained relatively constant during the next 24-h period. Because Mcl-1 protein level was significantly decreased at 6 h post-detachment and continued to decline during the 24 h period, this finding ruled out transcriptional regulation as responsible for the Mcl-1 downregulation. Moreover, the observation that proteasome inhibitors completely inhibited detachment-induced Mcl-1 downregulation strongly supported protein degradation and stabilization of Mcl-1 by Cav-1 as a key control mechanism.

Proteasomal degradation of a protein is generally triggered by its ubiquitination (Glickman and Ciechanover, 2002). We tested and found that Mcl-1 is ubiquitinated during cell detachment and that this process is inhibited by Cav-1. The mechanism by which Cav-1 inhibits Mcl-1 ubiquitination is unclear but likely involves steric hindrance of the ubiquitination sites by Cav-1. The interaction between Cav-1 and Mcl-1 may also affect Mcl-1 phosphorylation which has been linked to its ubiquitination. For example, phosphorylation of Mcl-1 at Ser159 by glycogen synthase kinase-3 was observed during

cell anoikis (Woods et al., 2007) and was found to promote Mcl-1 ubiquitination and subsequent degradation (Maurer et al., 2006).

# Conclusion

In conclusion, we reported the effect of reactive oxygen species, namely superoxide anion, hydrogen peroxide, and hydroxyl radicals on the lung cancer cells aggressive behaviors. Moreover, the role of nitric oxide inhibition of anoikis via Cav-1-dependent pathway was revealed. Importantly, we reported herein for the first time regarding role of Cav-1 in anoikis, cell migration, and invasion. While the role of Cav-1 in regulation of cell anoikis is unclear, our finding indicated that Cav-1 rendered lung cancer cell resistant to anoikis by interacting with anti-apoptotic Mcl-1 protein. We found that Cav-1 interacts with Mcl-1 and stabilizes the protein by blocking its ubiquitination and subsequent degradation. Because an elevated expression of Cav-1 and Mcl-1 has been linked to the progression of cancer and metastasis, the findings of this study could be beneficial to the understanding on cancer etiology and metastasis mechanisms.

## Suggestion for Further Works

The present study has suggested the role and implication of caveolin-1 protein in the regulation of processes in cancer cells metastasis which facilitates the further studies in animal model. Whether or not caveolin-1 expressed cells have more potential to establish metastatic colony in animals is of very interest. Also, the mechanisms as well as genes regulating different expression level of this protein are necessary to be investigated.

In addition, findings of the present study should be of important for prognosis prediction of lung cancer if the information regarding expression levels of caveolin-1 at the primary tumor and metastatic tumor are revealed.

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