藥學博士 學位論文

Loss of Caveolin-1 promotes stem-like traits in breast cancer cells

인체 유방암 세포에서 Caveolin-1 감소에 의한 암줄기세포 특성 발현 촉진

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Loss of Caveolin-1 promotes stem-like traits in breast cancer cells

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ABSTRACT

Loss of Caveolin-1 promotes stem-like traits in breast cancer cells

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Cancer stem cells (CSCs), a subset of cancer cells characterized by the property of self-renewal and differentiation, initiate tumor growth and promote chemo-/radiotherapy resistance, which are considered to be responsible for progression, recurrence and metastasis. Several intrinsic and extrinsic signaling pathways maintaining CSCs population have been explored. A prominent property of CSCs is the ability to undergo self-renewal division. The dysregulation of self-renewal

signaling may account for the regeneration of the tumor. Hence, understanding the signaling pathways for maintaining features of CSCs is likely to be important for developing targeted anticancer therapies.

Caveolin-1 (Cav-1) is a major protein of caveolae, which is flask-shaped invagination at cell membranes. Caveolae participate in various cellular functions, such as vesicle trafficking, cholesterol homeostasis, and tumor progression. Cav-1 is thought to regulate the activity of proteins, such as Src family kinases, H-Ras, protein kinase C, epidermal growth factor tyrosine kinase, extracellular signal-regulated kinase, and endothelial nitric oxide synthase involved in oncogenic signaling pathways. In this context, Cav-1 has been proposed as a potential therapeutic target for disrupting tumor progression and metastasis.

In the present study, I investigated a role for Cav-1 in regulating the stemness of human breast cancer (MDA-MB-231) cells. To investigate whether Cav-1 could be involved in modulating the stemness of breast cancer cells, tumorspheres were generated from adherent cells. Cav-1 expression was significantly lower in tumorspheres than in adherent cells. Further, the proportion of breast stem-like $CD44^{high}$ and $CD24^{low}$ cells was increased in Cav-1 knocked down MDA-MB-231 cells. Mechanistically, the silencing of Cav-1 resulted in the elevated expression of the well-known stemness-related genes, *Nanog*, *Oct 3/4*, and *Sox2* with concomitant

upregulation of *Bmi-1*, a representative self-renewal regulator. In line with above findings, the Cav-1 knock down increased the size and the number of spheres derived from MDA-MB-231 cells. In a CSC-like state, the reduced Cav-1 levels were attributable to its destabilization through ubiquitin-proteasome degradation. Src-mediated phosphorylation of Cav-1 at the Tyr 14 residue is essential for its degradation. The expression of p-Cav-1 (Tyr^{14}) and p-Src (Tyr^{416}) was markedly elevated in tumorsphere cells, compared to adherent cells. Y14F Cav-1 mutation by replacing Tyr^{14} with phenylalanine was sufficient to abolish phosphorylation and ubiquitination of Cav-1 protein. To verify that Cav-1 suppresses the breast cancer cell stemness, we overexpressed Cav-1 in MDA-MB-231 tumorspheres. As a result, the MDA-MB-231 tumorspheres overexpressing WT-Cav-1 exhibited the reduced ability of spheroid formation compared with Mock treated control cells. Notably, the tumorsphere-forming ability of cells expressing Y14F mutant Cav-1 was weaker than that of cells expressing WT-Cav-1. Likewise, the Y14F Cav-1 mutation significantly mitigated the inhibitory effects of Cav-1 on the expression of Bmi-1 and EMT markers. Taken together, these findings suggest that Cav-1destabilization by Src-mediated phosphorylation may play a pivotal role in manifestation and maintenance of stemness in breast cancer cells.

However, a question that still needs to be addressed is how Cav-1 destabilization promotes stemness properties. The Nuclear erythroid 2 p45-related factor-2 (Nrf2) is a key transcription factor that regulates the antioxidant and detoxification enzymes. Recently, it has been reported that Nrf2 signaling is involved in CSC-like properties of several types of cancer cells. Notably, Nrf2 has been reported to directly interact with Cav-1. In breast cancer, Cav-1 binds to Nrf2 and enhances its degradation in cytosol through ubiquitination. The reduction of the intracellular accumulation of Nrf2 by Cav-1 led to a decrease in stemness-related gene expression. When degradation of Cav-1 occurs through ubiquitin-proteasomal degradation, Nrf2 is dissociated from the Cav-1 complex. The liberated Nrf2 accumulates in the nucleus and enhances the expression of stemness-related genes, which promote the manifestation of CSC-like phenotypes. Thus, Cav-1 interact with Nrf2 in breast cancer and inhibits the Nrf2 signaling, thereby suppressing the manifestation of CSC-like properties.

Recently, natural products have attracted much attention for prevention and treatment of cancer. Resveratrol (*trans*-3,4',5-triydroxystilbene), a phytoalexin found in grapes and other food products, has been investigated with regard to its chemopreventive and chemotherapeutic activities in various cancer cells as well as in animal models. Nonetheless, the underlying mechanism by which resveratrol

regulates the signal transduction pathway involved in maintenance of CSCs and manifestation of their characteristics still remains to be largely unclear. Therefore, I investigated the effects of the resveratrol on breast cancer stem-like properties in the context of its modulation of Cav-1-mediated signaling. In this study, I found the inhibitory effect of resveratrol on the viability and migration capacity of tumorsphere MDA-MB-231 cells. Further, resveratrol significantly decreased the number and the size of MDA-MB-231 tumorspheres and also the proportion of $CD44^{high}/CD24^{low}$ cell population Mechanically, the expression of self-renewal signaling molecules and EMT markers was reduced by treatment of resveratrol. Notably, resveratrol treatment decreased expression of self-renewal and EMT markers, which was elevated by knockdown of Cav-1.

In conclusion, reduced Cav-1 accumulation depends on its destabilization through ubiquitin-proteasome degradation. After degradation of Cav-1, Nrf2 is dissociated from the Cav-1 complex and enhances the expression of stemness-related genes.. Resveratrol has an inhibitory effects on manifestation of Cav-1-mediated CSC-like properties. This study herein indicates that Cav-1 acts as a pivotal regulator in manifestation and maintenance of stemness in breast cancer cells and can be an important therapeutic target for breast cancer therapy.

Key words

Caveolin-1; Nrf2; Cancer stem cells; Signal transduction; Resveratrol

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LIST OF ABBREVIATIONS

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES

Breast cancer stem cell, BCSC

B lymphoma Mo-MLV insertion region 1, Bmi-1

Cancer stem cell, CSC

Caveolin-1, Cav-1

Caveolin scaffolding domain, CSD

Circulating tumor cell, CTC

Cycloheximide, CHX

Disseminated tumor cell, DTC

Dithiothreitol, DTT

Dulbecco′s modified Eagle′s medium, DMEM

Epidermal growth factor receptor, EGFR

Epithelial-mesenchymal transition, EMT

Enhanced chemiluminescent, ECL

Ethylenediaminetetraacetic acid, EDTA

Extracellular matrix (ECM)

Extracellular signal regulated kinase, ERK

Fetal bovine serum, FBS

Glyceraldehyde 3 phosphate dehydrogenase, GAPDH

Hematoxylin and eosin, H&E

Heme oxygenase-1, HO-1

Immunocytochemical analysis, ICC

Immunohistochemical analysis, IHC

Integrin, ITG

Mitogen-activated protein kinase, MAPK

Mesenchymal-epithelial transition, MET

Matrix metalloproteinase, MMP

Nitric oxide, NO

Nonidet P-40, NP-40

Nuclear factor erythroid-related factor 2, Nrf2

Phenylmethyl sulfonylfluoride, PMSF

Phosphate-buffered saline, PBS

Polyvinylidene difluoride, PVDF

Propidium iodide, PI

Phosphatidylinositol 3-kinase, PI3K

Resveratrol, RSV

Reverse transcription-polymerase chain reaction, RT-PCR

Roswell park memorial institute 1640, RPMI 1640

Signal transducer and activator of transcription 3, STAT3

Small interfering RNA, siRNA

Small ubiquitin-related modifier, SUMO

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE

Standard deviation, SD

Tris-buffered saline containing 0.1% tween 20, TBST

Wild-type, WT

Chapter I

Role of Caveolin-1 in cancer progression and metastasis

1. Introduction

Caveolins are the main integral proteins of caveolae, a flask-shaped invagination present at the plasma membrane [1]. Caveolae are very heterogeneous in normal and tumor cells, and they are most abundant in stromal cells, such as adipocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells [2]. Caveolins have amino-terminal and carboxy-terminal domains localized at the cytoplasmic face of the cell membrane (**Fig. 1-1 A**) [3]. Caveolins also contain a scaffolding domain, referred to as the Caveolin scaffolding domain (CSD), for binding to signaling proteins, making caveolae key regulators of signal transductions. Caveolins modulate functions of several signaling molecules, such as Src, eNOS and H-RAS, involved in cell proliferation and growth (**Fig. 1-1 B**) [4, 5]. Caveolins consist of the three principle members, Caveolin-1 (Cav-1), Caveolin-2 (Cav-2) and Caveolin-3 (Cav-3). Cav-1 is widely expressed in various cells, and Cav-2 shares a expression profile similar to Cav-1 as it requires Cav-1 for stabilization. However, Cav-3 is predominantly expressed in muscle cells [6]. Cav-1 and Cav-3 form homo-oligomers, and oligomerization is essential for caveolae biogenesis. Ablation of Cav-1 and Cav-3 causes a deficiency of caveolae in various cell types [7, 8]. Caveolae formation by Cav-1 and Cav-3 involves oligomerization and association with cholesterol-rich lipid-raft domains. Caveolins have multiple functions in cells besides formation of

caveolae. These include cholesterol homeostasis, vesicle trafficking, endocytosis, as well as regulation of signal transduction [10-11]. Cav-1 expression is sufficient and necessary to drive the formation of morphologically identifiable caveolae, making it the first protein marker of caveolae [9, 10]. Cav-1 belongs to a select group of proteins that function both as a tumor suppressor and as an oncogene commonly associated with enhanced malignant behavior, such as metastasis and multi-drug resistance [11].

Metastasis is the spread of tumor cells from the primary site to distant organs and their subsequent growth, and is the major cause of cancer-associated death [12-14]. Only few circulating tumor cells (CTCs) survive the immune surveillance and hemodynamic forces [15]. Surviving CTCs cells will colonize distant organs and become disseminated tumor cells (DTCs). Remarkably, most DTCs do not survive the initial colonization, whereas the rest may persist to reside in the secondary sites in a quiescent state (cellular dormancy) for many years, or progressively grow to form metastases [13, 16] (**Fig. 1-2**).

Epithelial-mesenchymal transition (EMT) facilitates development of highly invasive and mobile characteristics of cancer cells, thus enabling their dissemination from the primary site. Activation of the EMT, an also bestow cancer cells with high plasticity by acquiring stem-like traits. According to the model of cancer stem cells

(CSC), a small subpopulation of cancer cells is endowed with stem like-traits to promote cancer progression. These CSCs induce tumor initiation and metastasis [17]. Several studies have demonstrated relations among EMT, stemness, and the metastatic initiating potential of DTCs. Induction of EMT in transformed epithelial cells was shown previously to culminate in endowing cells with stem-like traits [18, 19]. These stem-like traits in transformed epithelial cells promoted the initiation of primary tumors and accelerated metastasis [20, 21]. Hence, the fluctuation between EMT/mesenchymal-epithelial transition (MET) state and CSC-like traits may dictate whether DTCs will remain dormant or emerge to metastasis. Therefore, understanding the mechanism of physical translocation is likely to be important for preventing metastasis in patients who are diagnosed with early cancer.

2. The role of Cav-1 expression in human cancer

2.1 Expression of Cav-1 in human cancer cells

Mounting studies have demonstrated controversial roles of Cav-1 expression in human cancer. There is no consistent profile of Cav-1 expression in cancer cells. Cav-1 is down-regulated in breast cancer [22-24], gastric cancer [25], and hepatic cancer [26], whereas the expression levels of Cav-1 are elevated, according to advanced tumor stage, high histological type and the metastasis of some human

cancer cells, including renal [27], pancreatic [28], esophagus [26, 29] and colorectal [30] cancer. Previous studies indicated a contradictory profile of the expression of Cav-1 in breast [22, 24], gastric [25, 31], hepatic [26] and oral [32] cancer. Sagara and colleagues investigated the mRNA and protein expression levels of Cav-1 in 162 cases of breast cancer and found that the mRNA and protein expression of Cav-1 was suppressed in breast cancer tissue compared with the normal tissues [33]. In gastric cancer, the positive staining of Cav-1 was higher in the advance gastric cancer group than in the early gastric cancer group, whereas the progressive downregulation of Cav-1 in gastric epithelial cells was found to correlate with gastric carcinogenesis [25]. Moreover, the Cav-1 mRNA expression in hepatitis B virus-related hepatocellular carcinoma (HCC) cells was found to negatively correlate with the tumor size, major venous invasion, single or multiple tumors, pTNM staging and factors associated with the prognosis of HCC, inconsistent with other studies [26]. Several studies have shown the downregulation of Cav-1 in cancer cells, such as pancreatic [29] and renal [34] cancer compared with normal tissues. Conversely, breast [33], ovarian [35], lung [36], and hepatic [26] cancer cells exhibit upregulated Cav-1 compared with the normal cells. Further studies are needed to demonstrate the mechanism of the different Cav-1 expression.

2.2 Dual role of Cav-1 in cancer

Cav-1 may function both as a tumor suppressor and as an oncogene, depending on the stage of oncogenic transformation and extent of tumor progression (**Fig. 1-3**).

2.2.1 Tumor suppressor

In some tissues, Cav-1 functions as a tumor suppressor. Cav-1 is highly expressed in differentiated or quiescent cells, including adipocytes, endothelia, smooth muscle cells, and Type I pneumocytes. Several studies have shown that induction of Cav-1 inhibits colony growth and induces apoptosis in transformed cells and breast cancer cells [37-39], suggesting a possible role for Cav-1 as a negative regulator of cell proliferation.

2.2.2 (Transformation) suppressor

Accumulating data reveal the inverse relationship between Cav-1 expression and transformation, suggesting that Cav-1 functions as a "transformation suppressor" gene. During the initial characterization of Cav-1, it was demonstrated that the level of residual Cav-1 inversely correlated with soft agar growth [39]. In addition, forced re-expression of Cav-1 abrogates anchorage-independent growth of transformed cells [37, 38, 40, 41].

2.2.3 Oncogene

In certain tumors, further progression into a metastatic or drug-resistant form has been attributable to re-expression of Cav-1. Up-regulation of Cav-1 in these tumors is thought to contribute to tumor cell invasiveness and resistance to anoikis, properties that are essential for tumor cell metastasis [38, 42]. Increased Cav-1 has also been associated with the development of drug resistance in tumors. In some tissues [33, 43], including prostate, Cav-1 is not normally expressed but, as tumor progresses, expression increases, which may account for enhanced tumor cell malignancy. Moreover, re-expression of Cav-1 in lung adenocarcinoma cells is sufficient to promote filopodia formation, cell migration and enhance the metastatic potential of these cells [44]. These findings, taken all together, suggest an oncogenic, prometastatic function for Cav-1.

2.3 Implication of Cav-1 expression in pathogenesis of human cancer

Several studies have demonstrated that loss of Cav-1 has been associated with poor outcomes in various tumor types, such as prostate, gastric and pancreatic cancer as well as melanoma [45, 46]. Mechanistically, low stromal expression of Cav-1 increases TGFβ1 expression and induces phosphorylation and activation of Akt [47].

Conversely, there is no consistent change in Cav-1 expression between cancer cells and their normal adjacent cells, and the effects of Cav-1 expression on tumorigenicity and aggressiveness vary widely among different cancer types [2, 48]. On the other hand, high Cav-1 expression is correlated with good clinical outcomes in head and neck cancer and extrahepatic biliary carcinoma cells [49]. In *in vivo* experiments, Cav-1-knockout mice show increased progression of colon, lung, and breast, as well as melanoma and non-melanoma skin cancers [50, 51]. Several distinct epithelial and stromal mechanisms seem to be co-operating in Cav-1-knockout mice. For example, cyclin D1 is upregulated and RB phosphorylation is increased in mammary cancer cells of $Cav1^{-/-}$ mouse mammary tumor virus (MMTV)-polyoma middle T (PyMT) transgenic mice [52]. Similarly, Cav-1 knockout mice have increased cyclin D1 upregulation in non-melanoma skin cancer, which is induced by chemical carcinogenesis [51].

3. The role of Cav-1 in invasion, migration and metastasis

Recent studies have indicated that cell invasion during tumor progression may be critically dependent on the acquisition of EMT features. The aim of this section is to summarize on molecular events associated with Cav-1 in progression, invasion, and

metastasis. The role of Cav-1 effectors and core regulators, and molecular pathways associated with Cav-1 in progression and metastasis will be discussed.

3.1 Cav-1 and EMT

Multiple lines of evidence support that Cav-1 mediates the invasion and metastasis of cancer which are accompanied by the EMT. Cav-1 can promote bladder cancer metastasis by inducing EMT which is associated with activation of phosphatidylinositol 3-kinase (PI3K)/AKT and upregulation of Slug expression [53]. Moreover, overexpressed Cav-1 increases vimentin expression, but decreases E-cadherin expression. This accompanies the change of EMT, which causes the increased the motility and invasiveness in hepatocellular carcinoma [54]. The reduced levels of Cav-1 in hypoxia increases epidermal growth factor receptor (EGFR) activation, leading to the activation of signal transducer and activator of transcription 3 (STAT3). This, in turn, results in the downregulation of E-cadherin and upregulation of mesenchymal markers, such as Slug, α-SMA, N-cadherin and vimentin, suggesting that Cav-1 can mediate the EMT and promote invasiveness in gastric cancer [54].

3.2 Cav-1 and Rho-GTPases

Accumulating studies have demonstrated that Rho-GTPases are likely to play a role in tumor metastasis and invasion [55, 56]. Previous studies have indicated the role of Cav-1 in regulating the activity of Rho-GTPases in various cancers. The association between Cav-1 and Rho-GTPases promotes tumor metastasis, which depends on the elevated expression of α5-integrin and the enhanced activation of Src and Ras [57]. Lin and colleague have reported that Cav-1 expression inhibits RhoC GTPase activation and subsequently activates the p38 mitogen-activated protein kinase (MAPK) leading to suppression of migration and invasion of primary pancreatic cancer cells [58].

3.3 Cav-1 and matrix metalloproteinase (MMP)

MMPs are a family of zinc-containing proteolytic enzymes that degrade various components of ECM [59]. Cav-1 may function as a negative regulator of metastasis by inhibiting MT4-MMP expression in colon cancer [60]. Moreover, overexpressed Cav-1 reduces the metastasis and invasion capacity of metastatic mammary tumor cells by inhibiting the activity of MMP-2 and MMP-9 [52]. Conversely, the migration and invasion-promoting effect of Cav-1 overexpression in HCC appears

to be mediated by increasing secretion or expression of MMP-2, MMP-9 and MT1-MMP, as well as inducing an EMT-like phenotype [61].

4. Conclusion

As a main component of caveolae, Cav-1 is involved in many biological processes that include substance uptake and transmembrane signaling. In addition, Cav-1 contributes to cell transformation, tumorigenesis, and metastasis. As depicted in the **Fig. 1-3**, Cav-1 may function both as an oncogene and as a tumor suppressor depending on the stage of tumor progression. Because CSCs contribute to development and progression in cancer, the presence of CSC population in precancerous stage is an early indicator of malignant progression. Despite a number of controversies on role of Cav-1 in cancer, the majority of reports suggest that Cav-1 represents an important cancer cell biomarker in carcinogenesis, differentiation, metastasis and tumor progression, and independently serves as a predictor of overall survival rate. In addition, through interaction with other biological molecules, Cav-1 modulates stem-like traits. To succeed in establishing novel diagnostic molecular and targeted therapies against Cav-1, mechanical studies are required to further unveil the clinical value of Cav-1 expression in multiple types of cancer and cancer stem cells.

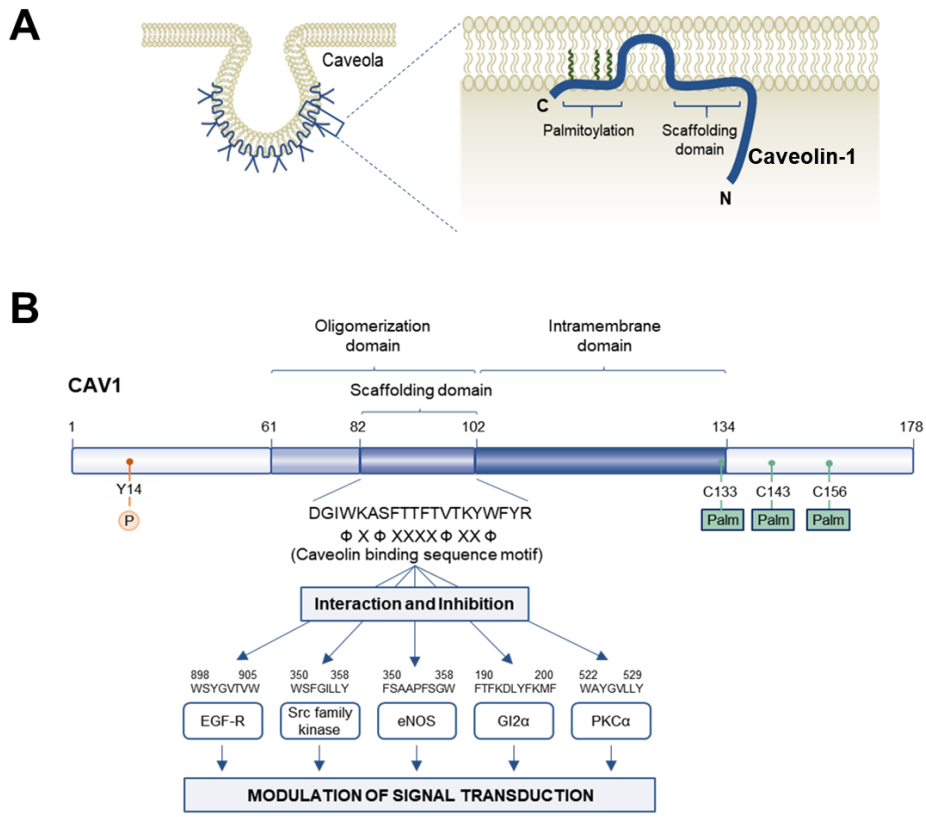

Figure 1-1. Structures of Caveolae and Caveolin-1. (A) The diagrams of Caveolae and Caveolin-1 . Cav-1 is inserted into the caveolar membrane, with the N and C termini facing the cytoplasm and a intramembrane domain embedded within the membrane bilayer. **(B)** The sequence of the caveolin-scaffolding domain (CSD; residues 82-102) and the caveolin binding sequence motifs within several caveolae-localized signaling molecules are shown. These include epidermal growth factor receptor (EGF-R), Src family tyrosine kinases, eNOS, G-protein α subunits (Gi2α), and PKC isoforms (PKCα). In most cases, this caveolin interaction is inhibitory,

leading to inactivation of the signaling molecules and modulation of downstream signal transduction.

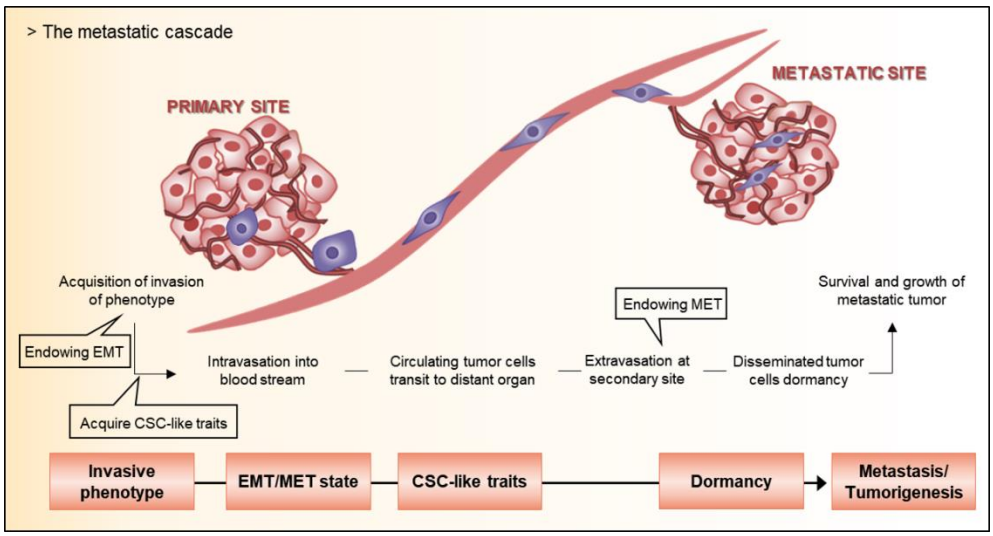

Figure 2-2. The metastatic cascade. Cancer cells within the primary tumor undergo an epithelial-mesenchymal transition (EMT) process and acquire stem-like traits (CSCs) and endow invasive capacity, then intravasate into the tumor vasculature in the form of circulating tumor cells (CTCs), which must be able to survive the circulating blood and evade from the innate immune response and other defenses. Once CTCs migrate to a secondary site, the settlement in supportive niches enables them to survive and retain their stem-like tumor-initiating capacity. In the target site, disseminated cancer cells (DTCs) encounter inhibitory signals resulted in the arrested in cell cycle subsequently leading to dormancy from months to decades while they adapt to their newfound microenvironment. Cancer cells undergo MET in order to acquire feature proliferation to metastatic outgrowth in the target site.

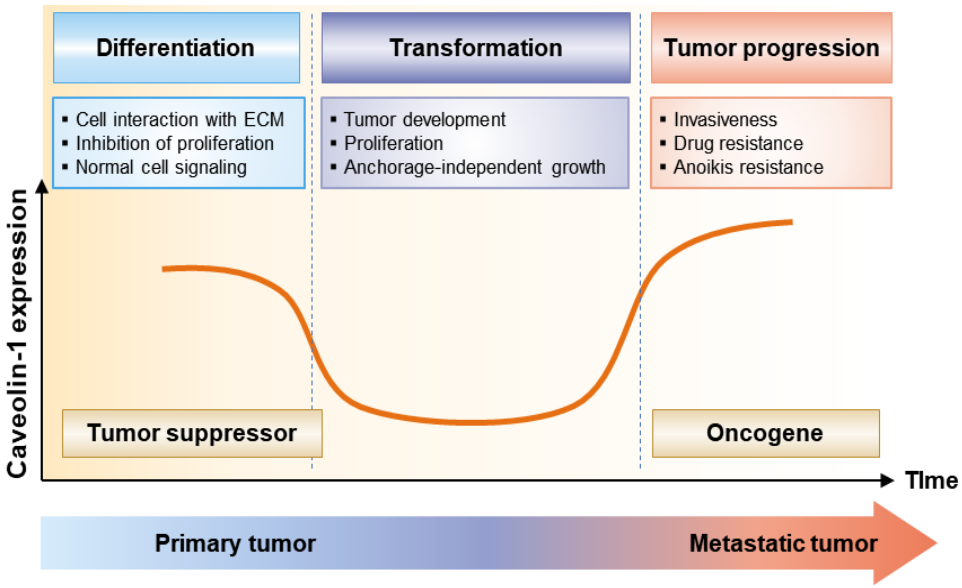

Figure 3-3. Dual role of Caveolin-1 in cancer. Cav-1 may function both as a tumor suppressor and as an oncogene, depending on the stage of oncogenic transformation and extent of tumor progression. Cav-1 is expressed at relatively high levels in many differentiated cells. During oncogenic transformation, Cav-1 is downregulated, in certain tumors, further progression into a metastatic or drug-resistant form is associated with re-expression of Cav-1. Upregulation of Cav-1 in these tumors is thought to contribute to tumor cell invasiveness and resistance to anoikis, properties that are essential for tumor cell metastasis. Increased Cav-1 has also been associated with the development of drug resistance in tumors.

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STATEMENT OF PURPOSE

Caveolin-1 (Cav-1), a major component of cell membrane caveolae, is involved in a variety of cellular signal pathway and transmembrane transport. Cav-1 acts as a scaffolding protein, modulating the transduction of multiple signaling molecules. To date, most of these signals are highlighted in cancer development. However, the molecular mechanism by which caveolin-1 is involved in regulating cancer stem cell signaling networks remain largely unknown. Because CSCs contribute to development and progression in cancer, the presence of CSC population in precancer stage is an early indicator of malignant progression. In the present study, I thus elucidated the role of Cav-1 in breast CSC-like model. Furthermore, I investigated the effect of Cav-1and Nrf2 binding on stemness signaling transduction. Subsequent work confirmed the effect of resveratrol on the stemness phenotype and underlying molecular mechanisms in the context of inhibiting of migration and stemness in breast cancer cells. To establish novel diagnostic molecular and targeted therapies against Cav-1, I unveiled the mechanical studies about the value of Cav-1 expression in types of breast cancer and cancer stem cells.

Chapter II

Src-mediated phosphorylation, ubiquitination and degradation of Caveolin-1 promotes breast cancer cell stemness and progression

1. Abstract

Cancer stem cells (CSCs) are responsible for tumor initiation, metastasis and recurrence. Caveolin-1 (Cav-1) is a major protein of caveolae, which participates in various cellular functions, such as vesicle trafficking, cholesterol homeostasis, tumor progression, etc. In the present study, I investigated a role for Cav-1 in regulating the stemness of human breast cancer (MDA-MB-231) cells. Cav-1 expression was significantly lower in tumorspheres than in adherent cells. The silencing of Cav-1 enhanced stemness of MDA-MB-231 cells. Mechanistically, Cav-1 silencing was accompanied by enhanced expression of Bmi-1, which is a representative self-renewal regulator, and promoted epithelial-mesenchymal transition. In a CSC-like state, reduced Cav-1 depends on its destabilization through ubiquitin-proteasome degradation. I further found that Src-mediated phosphorylation of Cav-1 at the Tyr 14 residue is essential for its degradation. Taken together, these findings suggest that Cav-1 destabilization by Src may play a pivotal role in manifestation and maintenance of stemness in breast cancer cells.

Keywords: breast cancer stem cell; Caveolin-1; c-Src; phosphorylation; destabilization;

2. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide. In spite of the diverse therapeutic options, chemo-/radioresistance and disease relapse often develop, which is attributed to the presence of stem-like cancer cells in the tumor microenvironment [1, 2].

Cancer stem cells (CSCs) are defined as a subset of cancer cells characterized by the property of self-renewal and differentiation, which drive tumorigenesis and tumor heterogeneity [3-5]. Several lines of evidence suggest that epithelial-mesenchymal transition (EMT) and stem cell-like traits are intertwined processes to foster metastatic tumor microenvironment. As an initial step of tumor cell migration, EMT can induce differentiation of cancer cells into a CSC-like state [6]. In this context, CSCs may underlie local and distant metastases by acquiring mesenchymal features which would greatly facilitate systemic dissemination from the primary tumor mass to metastatic tumor [7]. Many of the signaling molecules including Wnt/β-catenin, Notch, Hedgehog, STAT3, and TGF-β are involved in generation and maintenance of CSCs [8-10]. Therefore, therapeutic strategies targeting CSCs by modulating these signaling molecules have attracted special attention.

Caveolin-1 (Cav-1) is a major protein of caveolae, which is flask-shaped invagination at cell membranes. Caveolae participate in various cellular functions,

such as vesicle trafficking, cholesterol homeostasis, tumor progression, and especially modulation of various signal transduction pathways [11, 12]. Cav-1 is thought to regulate the activity of proteins, such as Src family kinases, H-Ras, protein kinase C, epidermal growth factor tyrosine kinase, extracellular signal-regulated kinase, and endothelial nitric oxide synthase involved in oncogenic signaling pathways [13, 14]. Src interaction with the plasma membrane is an important determinant of its activity. For instance, the intrinsic kinase activity of Src phosphorylates Cav-1. Subsequent binding of the activated Src to phosphotyrosylated Cav-1 modulates its association with the membrane [15]. Originally identified as a substrate for v-Src, Cav-1 is phosphorylated on Tyr14 by c-Src [16]. Circumstantial evidence suggests that phosphorylated Cav-1 also regulates its cellular localization and function [17-21]. Collectively, Cav-1 modulates various signaling pathways and elicits anti-cancer responses in breast [22], glioblastoma [23], lung [24], and other cancer types [25].

The role of Cav-1 in development and progression of cancer is controversial because it is suggested to exert both tumor-suppressive and oncogenic effects. In recent studies, Cav-1-mediated signaling has been correlated with maintenance of stemness to augment various cancer stem cells expansion [26]. However, the precise function of Cav-1, particularly in relation

to its phosphorylation by Src, in affecting CSCs is largely unknown. Here I report that Src-mediated phosphorylation and subsequent destabilization of Cav-1 contributes to maintenance of breast CSCs and manifestation of their characteristics.

3. Materials and methods

Reagent and antibodies

Dulbecco's modified Eagle's medium (DMEM), Rosewell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). TRIzol® was obtained from Invitrogen (Carlsbad, CA, USA). Primary antibodies for Bmi-1, Notch-1, CD133, Sox-2, Src, p-Src, and ubiquitin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Cav-1, Oct 3/4, Snail, and β-actin were obtained from Santa Cruz (Santa Cruz, CA, USA). Antibodies against p-Cav-1, Twist1, N-cadherin, E-cadherin, CD24 and CD44 were purchased from BD Biosciences (Bedford, MA, USA). The bicinchoninic acid (BCA) protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL, USA). MG-132 was obtained from Enzo Life Sciences (Exeter, UK). The Src inhibitor (PP2) was purchased from EMD Milipore Corporation (Bilierica, MA, USA).

Cell culture

The human breast cancer cell lines MCF-7, T47D, SKBR3, MDA-MB-453, MDA-MB-231, and MDA-MB-468 were obtained from the Korean Cell Line Bank (Seoul,

South Korea). MDA-MB-453, MDA-MB-231 and MDA-MB-468 cells were maintained in DMEM, whereas MCF-7, T47D, and SKBR3 cells were maintained in RPMI 1640 cell culture medium. All culture media were supplemented with 10% FBS and 1 % antibiotics and cells were grown at 37 ℃ with 5 % CO_2 /95 % air.

Tumorsphere culture

For tumorsphere formation from the adherent cells, single cells were cultured in a serum free DMEM/F12 medium supplemented with B27 (GIBCO), 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL basic fibroblast growth factor (b-FGF; PeproTech, Rocky Hill, NJ, USA) and 4 ng/mL heparin (Sigma-Aldrich). Primary tumorspheres were seeded at a density of 1 x 10^4 cells/mL in 100 mm ultralow attachment plates (Corning, NY, USA) for 5 consecutive days, and 2 mL of medium was added every third day. To culture secondary tumorspheres, primary tumorspheres were gently collected and dissociated into a single-cell suspension using 40 μm strainer. Single cells were counted and then seeded again for another 5 days with addition of 2 mL medium every third day. Using the same experimental method, tertiary mammospheres were generated from secondary mammospheres. The number of mammospheres formed (> 100 μm) was counted at indicated times under a microscope. Images were

analyzed by using the ImageJ software (http://rsb.info.nih.gov/ij/docs/index.html). 3D picture analysis and quantification were carried out using the ReViSP software (https://sourceforge.net/projects/revisp/).

Flow cytometry analysis

Cells were collected, washed with phosphate-buffered saline (PBS), and dissociated with Accutase solution (Sigma-Aldrich). Cells were then counted and washed with PBS containing 2 % FBS and 0.1% Tween-20. Cells were stained with CD24-PE and CD44-APC for 30 min at 4 °C. After incubation, cells were collected and washed with PBS again. Cells were dissociated into single cells by using 40 µm strainer, and then the population of $CD44^{high}/CD24^{low}$ cells was measured using BD FACSCalibur (Becton Dickinson Biosciences, San Jose, USA).

ALDEFLUOR Assay

The ALDEFLUOR™ kit (StemCell Technologies, Durham, NC, USA) was used to identify the cells that express high levels of the enzyme aldehyde dehydrogenase (ALDH). MDA-MB-231 mammospheres were obtained and suspended in the ALDEFLUOR Assay Buffer containing ALDH substrate at a density of 1 x 10^5 cells/mL and incubated for 30 min at 37 °C. For negative control, each sample was

treated with an ALDH specific inhibitor, diethylaminobenzaldehyde (DEAB), for background fluorescence. The sorting gates were established by eliminating the cells stained positive with ALDH in negative control group. Data were analyzed by the BD FACSCalibur (Becton Dickinson Biosciences).

Western blot analysis.

Whole cell lysate was prepared by scapping the cells in RIPA lysis buffer [(150 mM NaCl, 0.5 % Triton x 100, 50 mM Tri-HCl (pH 7.4), 25 mM NaF, 20 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 0.1 mM phenylmethane sulfonyl fluoride (PMSF)] for 15 min on ice followed by centrifugation at 13000 *g* for 15 min. The supernatant containing proteins was collected and stored at -70 ℃. For Western blot analysis, the protein concentration of whole cell lysates was measured by using the BCA protein assay kit (Pierce, Rockford, IL, USA). Lysates from cells were separated by running through 8-12 % SDS-PAGE gel and transferred to the polyvinylidene difluoride (PVDF) membrane (Gelman Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5% non-fat dry milk/TBST (Tris-buffered saline buffer containing 0.1 % Tween-20) for 1 h at room temperature. The membranes were incubated with the respective primary antibody diluted in TBST overnight 4 ℃. Blots were rinsed three times with TBST at 10-min intervals followed by incubation with respective

horseradish peroxidase conjugated secondary antibodies (rabbit, mouse or goat) in TBST for 1 h at room temperature. The blots were washed again three times with TBST. The band intensities in Western blotting were visualized with an enhanced chemiluminescent (ECL) detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and quantified with the LAS-4000 Image Analyzer (Fujifilm, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was isolated from each cell by using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To generate the cDNA from RNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) for 50 min at 42 °C and again for 15 min at 72 °C. About 1 μL of cDNA was amplified with a PCR mixture (HS Prime Taq 2X Premix, Daejeon, South Korea) in sequential reactions. The primers used for each RT-PCR reactions are as follows : *CAV-1*, 5'-ATG TCT GGG GGC AAA TAC GTA-3' and 5'–TTG GAA CTT GAA ATT GGC ACC A-3' ; *BMI-1*, 5'-CCA GGG CTT TTC AAA AAT-3' and 5'-GCA TCA CAG TCA TTG CTG CT-3' ; *NOTCH-1*, 5'-GGG TCC ACC AGT TTG AAT GG-3' and 5'-GTT TGC TGG CTG CAG GTT CT-3'; *GAPDH*, 5'-AAG GTC GGA GTC AAC GGA TTT-3'

and 5’-GCA GTG AGG GTC TCT CTC T-3’; *TWIST1*, 5’-GGA GTC CGC AGT CTT ACG AG-3 and 5’-TCT GGA GGA CCT GGT AGA GG-3’ ; *SNAIL,* 5’-CCT GCT GGC AGC CAT CCC AC-3’ and 5’-GGC ACG GTG TGG CTT CGG AT-3’ ; *SLUG,* 5’-ACG CCC AGC TAC CCA ATG GC-3’ and 5’–AGG GCG CCC AGG CTC ACA TA-3’ ; *ZEB1*, 5’-AGT GAT CCA GCC AAA TGG AA-3’ and 5’-TTT TTG GGC GGT GTA GAA TC-3’ (forward and reverse, respectively). Amplification products were analyzed by 1.5-2 % agarose gel electrophoresis, followed by staining with SYBR Green (Invitrogen, Carlsbad, CA, USA) and photographed using fluorescence in LAS-4000 (Fujifilm, Tokyo, Japan).

Transient transfection of siRNA

MDA-MB-231 cells were seeded at a density of 1 x 10^5 cells/mL in 100 mm dish in complete growth media. Cav-1 siRNA (25 nM) was transfected into MDA-MB-231 cells with lipofectamine RNAiMAX (Invitrogen) reagent according to the manufacturer’s instructions. The target sequence for human Cav-1 siRNA was 5’ -AGA CGA GCU GAG CGA GAA GCA UU3’ (forward) and 5’ -UGC UUC UCG CUC AGC UCG UCU UU-3’ (reverse). siRNA oligonucleotides targeting for Cav-1 was purchased from Genolution Pharmaceuticals (Seoul, South Korea).

Transient transfection of plasmid

Transient transfection of plasmid encoding native or mutant Cav-1 in which tyrosine 14 is replaced by phenylalanine (Y14F-Cav-1) was performed by Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 36 h transfection, cells were harvested or cultured to generate according to purpose of the experiment.-

Immunofluorescent analysis

Human paraffin-embedded breast cancer tissue array (US Biomax, Inc., cat. no. BR1201a; Rockville, MD, USA) was subjected to deparaffinization with xylene. Following antigen retrieval by heated citrate buffer, sections were permeabilized and blocked according to the standard protocol. After overnight incubation at 4 °C with anti-Cav-1, the tissue sections were washed with PBS and then labeled with TRITC-conjugated anti-mouse IgG secondary antibody for 1 h at room temperature. The slides were then analyzed under a fluorescent microscope.

Immunocytochemical analysis

MDA-MB-231 cells were plated on the 8-well chamber slide (0.5 x cells/well) and transfected with control or Cav-1 specific siRNA. Cells were fixed in 95% methanol

for 10 min at -20 ℃. After rinse with PBS containing 0.1 % Tween 20 (PBST), cells were incubated in 0.2 % Triton X-100 in PBS for 5 min. After three washing steps with PBST, cells were blocked for 2 h in fresh blocking buffer [PBST (pH 7.4) containing 5% bovine serum albumin (BSA)]. Dilution (1:100) of primary antibody was made in PBST with 1% BSA, and cells were incubated overnight at 4 ℃. After three washing steps with PBST, the cells were incubated with a diluted (1:1000) TRITC-conjugated anti-mouse or FITC-conjugated anti-rabbit IgG secondary antibody in PBST with 1% BSA at room temperature for 1 h. Cells were also stained with 4',6-diamidino-2-phenylindole (DAPI) and rinsed with PBST. Stained cells were analyzed under a confocal microscope (Leica Microsystems, Heidelberg, Germany) and photographed. The accumulation effects area was quantified using the Image J software.

Immunoprecipitation

Cells were lysed in 250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 μM NaF, 2 μM sodium orthovanadate, 1 mM PMSF and 10 mM N-ethylmaleimide. Total protein (500 μg) was subjected to immunoprecipitation by shaking with Cav-1 primary antibody at 4 ℃ for 24 h followed by the addition of 20 μL of 25 % protein G-agarose bead slurry (Santa Cruz Biotechnology, Inc.; Santa

Cruz, CA, USA) and additional shaking for 2 h at 4 °C. After centrifugation at 10,000 *g* for 1 min, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. After final wash, immunoprecipitate was resuspended in 50 μL of 2X SDS electrophoresis sample buffer and boiled for 5 min. Forty five μL of supernatant from each sample was loaded on SDS-PAGE. The expression of phosphorylated or ubiquitinated Cav-1 and phosphorylated Src was visualized by antibody against p-Src, p-Cav-1 or ubiquitin.

Site-directed mutagenesis

Point mutation of tyrosine to phenylalanine at the residue 14 (Y14F) in Cav-1 was induced by using a QuikChange® site-directed mutagenesis kit (Stratagene; Cedar Creek, TX, USA) according to the manufacturer's protocol. Mutant strand synthesis reaction was performed by denaturation at 96 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 8 min with 30 cycles using the 5.4 kb plasmid template encoding Myc-Cav1. Generally, extension time of 1 min/kb is recommended depending on the length of plasmid template and extension time of 8 min was set as an optimal condition. The following complementary primer pairs were used: sense, 5′-GAC TCG GAG GGA CAT CTC TTC ACC GTT CCC ATC CGG G-3′; antisense, 5′-CCC GGA TGG GAA CGG TGA AGA GAT GTC CCT CCG AGT C-3′. The

DNA sequences of all plasmids were verified by sequencing (Cosmo Genetech, Seoul, South Korea).

Tumorigenesis assay

All animal experiment were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (authorization number: SNU-160912-1). Six-week-old female BALB/c nude mice (total 12) were purchased from Central Lab Animal, Inc. (Seoul, South Korea). All the animals were maintained under specific pathogen-free (SPF) conditions with 12-h light/12-h dark cycle. After one week of acclimation period, 1×10^4 mock, native (WT-Cav-1) or mutant (Y14F-Cav-1) transfected MDA-MB-231 tumorspheres re-suspended in equal volumes of PBS and matrigel (total volume of 100 μL) were injected into the fourth mammary fat pads of mice. Four mice were included in each group. The tumor incidence was identified by palpation every 3 day. Tumor volume was regularly measured with digital calipers and calculated according to the formula; $V = 0.5\ ab^2$, where 'a' is the longest and 'b' is the shortest perpendicular diameters. After mice were killed, xenograft tumors were excised and fixed in formalin for further analysis.

Statistical analysis

Data were represented as means of ± standard deviation (SD) at least three independent experiments. Statistical significance was determined by Student's *t*-test and a *p*-value of less than 0.05 was considered to be statistically significant.

4. Results

Cav-1 is down-regulated in tumorspheres derived from highly invasiveness breast cancer cells

I initially investigated the correlation between Cav-1 expression and clinical progress of breast cancer patients. For this purpose, immunofluorescent analysis was performed to detect the expression of Cav-1 in 120 patients tissues with different clinical stages, including stage 1 (n = 6), stage 2 (n = 94), and stage 3 (n = 20). As shown in **Fig. 2-1A**, Cav-1 expression was strongly correlated with clinical stages in breast cancer patients. This finding is corroborated by the microarray data retrieved from the Cancer Genome Atlas analyzed through the oncomine web portal (www.oncomine.org) (**Fig. 2-1B**). Likewise, higher expression of Cav-1 was found in MDA-MB-231 and MDA-MB-468 cells, highly invasiveness breast cancer cell lines (**Fig. 2-1C**). Next, I examined whether Cav-1 could be involved in modulating the stemness of breast cancer cells. I enriched for stemness characteristics of breast cancer cells (MCF-7, MDA-MB-453, MDA-MB-231 and MDA-MB-468), by culturing them as spheroids (**Fig. 2-1D**). As shown in **Fig. 2-1E**, the expression of CD133, Oct 3/4, and Sox2, well-known stemness markers, was increased in tumorspheres. I also observed that tumorspheres derived from MDA-MB-231 and MDA-MB-468 breast cancer cells exhibited much lower levels of Cav-1 (**Fig. 2-1F).**

Furthermore, immunofluorescent analysis reveals that Cav-1 expression is associated with a stemness marker, Sox2 (**Fig. 2-1G**). Based on these observations, Cav-1 is likely to play a crucial role in the stemness of breast cancer cells.

Silencing of Cav-1 enhances the stemness phenotype of MDA-MB-231 cells

As Cav-1 expression is down-regulated in MDA-MB-231 tumorspheres compared to adherent cells, I carried out an experiment using siRNA to examine the impact of Cav-1 on manifestation of stemness. As shown in **Fig. 2-2A**, the silencing of Cav-1 resulted in the elevated expression of the well-known stemness-related proteins, Nanog, Oct 3/4, and Sox2. Consistently, the Cav-1 knockdown increased the size and the number of spheres in MDA-MB-231 (**Fig. 2-2B).** In addition, the proportion of $CD44^{high}$ and $CD24^{low}$ cells was increased when Cav-1 was knockdown in MDA-MB-231 (**Fig. 2-2C**). The ALDH activity, which also accounts for major stemness property, was increased by transfection with the Cav-1 siRNA (**Fig. 2-2D**). These findings, taken all together, suggest that Cav-1 plays an important role in maintaining a stemness of breast cancer cells.

Loss of Cav-1 increases the expression of a self-renewal marker, Bmi-1 and EMT markers

As described above, silencing of Cav-1 results in enhancement of stemness in MDA-MB-231 breast cancer cells. Self-renewal is one of the important properties employed by the CSCs to maintain the proliferating capacities. Notch1 and Bmi-1 are known as the key regulators of self-renewal activity in mammary stem cells [27]. In this study, Cav-1 knockdown up-regulated Bmi-1 expression at both mRNA and protein levels, but there was no change in the expression levels of Notch-1 (**Fig. 2-3A, B**). Since acquisition of stem-like traits has been linked to EMT properties [28, 29], I also investigated the role of Cav-1 in the induction of EMT in MDA-MB-231 cells. The induction of EMT leads to the loss of epithelial characteristics (such as E-cadherin, desmoplakin and claudins) and an acquisition of mesenchymal phenotype (vimentin, α5β1integrin and fibronectin) [30, 31]. Cav-1 inhibition increased the expression of EMT marker genes, such as *Snail, Twist1,* and *N-Cadherin* (**Fig. 2-3C**) and their protein products (**Fig. 2-3D**). An immunocytochemistry assay verified the upregulation of N-cadherin and snail in Cav-1 knockdown cells (**Fig. 2-3E**). Moreover, I found that elevated expression of α5 and β1 integrins were observed in tumorspheres compared to adherent cells. I next determined whether Bmi-1 could affect EMT signaling. siRNA-mediated silencing of Bmi-1 markedly reduced the

mRNA and protein expression of EMT markers in MDA-MB-231 cell (**Fig. 2-3G** and **3H**). These results suggest that Bmi-1 expression induced by the Cav-1 downregulation plays a role in stimulating EMT in a MDA-MB-231 breast cancer cells.

Expression of the Cav-1 protein, but not its mRNA transcript, is reduced and induces its proteasomal degradation in MDA-MB-231 tumorspheres

In contrast to no significant difference in Cav-1 mRNA expression levels between the adherent and the tumorsphere cells, MDA-MB-231 derived tumorspheres exhibited significant alteration in the steady state level of the Cav-1 protein (**Fig. 2-4A**). To determine whether acquisition of stemness is a consequence of Cav-1 protein destabilization, I monitored the degradation of Cav-1 in tumorsphere as well as adherent cells after inhibition of *de novo* protein synthesis by cycloheximide. As illustrated in **Fig. 2-4B**, Cav-1 in the MDA-MB-231 tumorspheres underwent degradation rapidly as compared with adherent cells after addition of cycloheximide. Many labile proteins are commonly degraded via the ubiquitin-proteasomal pathway. As shown in **Fig. 2-4C**, treatment of MDA-MB-231 tumorspheres with the proteasome inhibitor, MG-132 resulted in increased accumulation of Cav-1. Generally, proteins subjected to proteasomal degradation are

marked by prior attachment of ubiquitin to their lysine residue. As illustrated in **Fig. 2-4D,** MDA-MB-231 tumorspheres exhibited enhanced ubiquitiation of Cav-1 with a concomitant acquisition of stem-like properties compared to adherent cells.

Role of Src in phosphorylation and stability of Cav-1 in MDA-MB-231 tumorspheres

Src has a kinase activity which contributes to its association with Cav-1. Thus, binding of Src to phosphotyrosylated Cav-1 affects Cav-1-regulated signaling [12, 32]. This prompted us to examine whether Cav-1-Src interaction was involved in the maintenance of stemness in breast cancer cells. As shown in **Fig 2-5A**, the expression of p-Cav-1 (Tyr^{14}) and p-Src (Tyr^{416}) was much higher in tumorsphere cells, compared to adherent cells. In line with the notion that Src-dependent phosphorylation targets Cav-1 for degradation, PP2, a specific Src inhibitor, abrogated Cav-1 phosphorylation in MDA-MB-231 tumorspheres (**Fig. 2-5B)**. These findings suggest that sustained phosphorylation of Cav-1 (Tyr^{14}) by active Src accounts for Cav-1 degradation. Moreover, PP2 treatment attenuated Cav-1 ubiquitination (**Fig. 2-5C**), the characteristic signature of proteasomal targeting. Consistently, the Cav-1 expression repressed in MDA-MB-231 tumorspheres was restored after addition of PP2, whereas the expression levels of Cav-1 in the adherent

cells had no differences (**Fig. 2-5D**). In addition, treatment of MDA-MB-231 tumorspheres with PP2 decreased the size and the number of spheres (**Fig. 2-5E**). Thus, it is likely that destabilization of Cav-1 in tumorsphere occurs by Src-dependent phosphorylation of Cav-1 (Tyr^{14}).

Tyrosine 14 of Cav-1 is a key amino acid in maintaining stemness of breast cancer

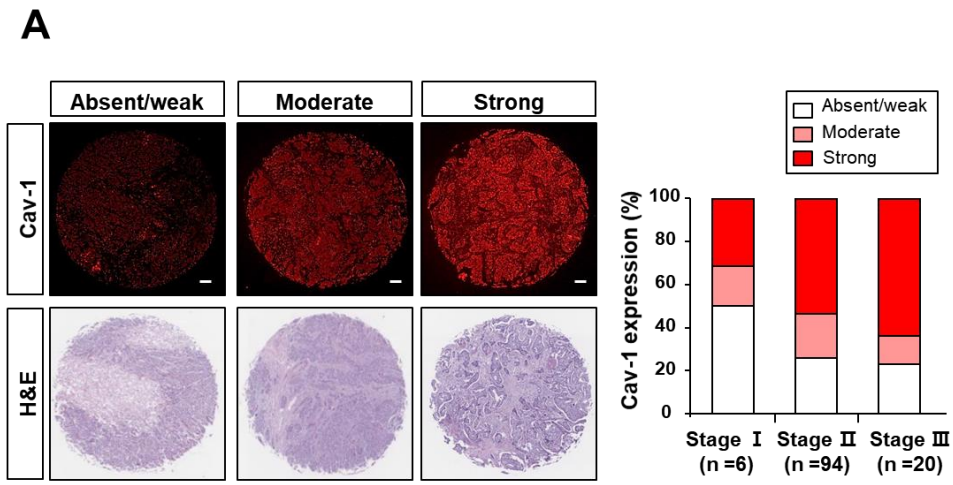
The tyrosine 14 residue of Cav-1 is thought to be the principal site for recognition by c-Src kinase. To ensure this tyrosine residue is implicated in the regulation of Cav-1, I mutated the tyrosine 14 to phenylalanine, which eliminated the phosphorylation site. The mutant construct (Y14F-Cav-1) was then transfected into MDA-MB-231 cells. Analysis of each of the transfected lines revealed that the Y14F-Cav-1 mutation was sufficient to abolish phosphorylation and ubiquitination of Cav-1 expression (**Fig. 2-6A** and **6B**). To verify that Cav-1 suppresses the breast cancer cell stemness, I overexpressed Cav-1 in MDA-MB-231 tumorspheres. As a result, the MDA-MB-231 tumorspheres overexpressing WT-Cav-1 reduced the ability of spheroid formation compared with MOCK cells. Additionally, the tumorsphere-forming ability of cells expressing Y14F mutant Cav-1 was weaker than that of cells expressing WT-Cav-1 (**Fig. 2-6C**). Consistent with this finding, the Y14F-Cav-1 mutation significantly mitigated the retarding effects of Cav-1 on the

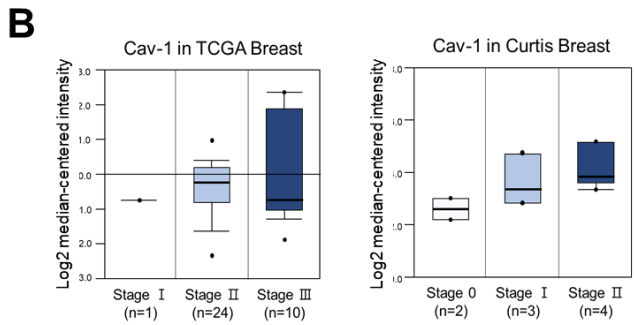
expression of Bmi-1 and EMT markers (**Fig. 2-6D** and **6E**). Thus, tyrosine 14 is considered a key amino acid within Cav-1 that regulates stemness capacity in breast cancer cells.

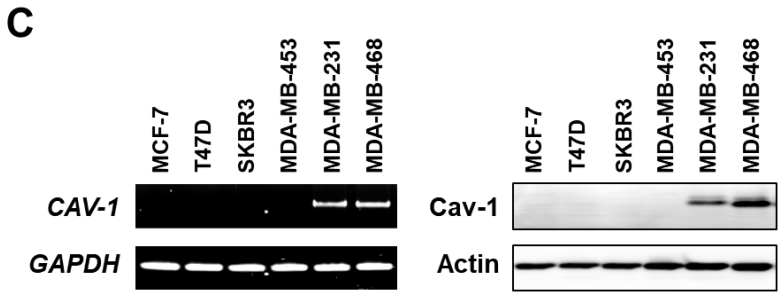
Tyrosine 14 of Cav-1 is essential for the tumorigenicity of MDA-MB-231 tumorspheres

After finding that phosphorylation of tyrosine 14 of Cav-1 is pivotal to the stem-like properties of breast cancer cells *in vitro*, I assessed whether this amino acid residue would affect the oncogenicity of MDA-MB-231 tumorspheres. The tumorigenic ability was measured for MDA-MB-231 tumorspheres, which were transfected with MOCK, WT-Cav-1 or Y14F-Cav-1 mutant construct and inoculated into the mammary pads of BALB/c nude mice. The representative image of the excised tumors at the day 40 after inoculation was presented in **Fig. 2-7A**. As shown in **Fig. 2-7B**, the tumors derived from tumorspheres harbouring Y14F-Cav-1 appeared to have longer latency than those from MOCK and WT-Cav-1 tumorsphere groups. After 40 days, the volume and the weight formed in the Y14F-Cav-1 tumorsphere group were markedly reduced compared with those in MOCK and WT-Cav-1 tumorsphere groups **(Fig. 2-7C** and **7D)**. Hematoxylin-eosin (H&E) staining showed the presence of prominent apoptotic figures in MOCK tumorsphere groups,

in contrast to tumors isolated from WT-Cav-1 tumorsphere groups and Y14F-Cav-1 tumorsphere groups containing less apoptotic cells. Immunohistochemical analysis also showed a relatively weaker nuclear staining of pCav-1 in the tumor tissues of mice transplanted with Y14F-Cav1 spheres **(Fig. 2-7E)**.


A
Absent/weak
Moderate
Strong
Cav-1
H&E
Absent/weak
Moderate
Strong
Cav-1 expression (%)
100
80
60
40
20
0
Stage Ⅰ (n =6)
Stage Ⅱ (n =94)
Stage Ⅲ (n =20)


B
Cav-1 in TCGA Breast
Log2 median-centered intensity
Stage Ⅰ (n=1)
Stage Ⅱ (n=24)
Stage Ⅲ (n=10)
Cav-1 in Curtis Breast
Log2 median-centered intensity
Stage 0 (n=2)
Stage Ⅰ (n=3)
Stage Ⅱ (n=4)


C
MCF-7
T47D
SKBR3
MDA-MB-453
MDA-MB-231
MDA-MB-468
CAV-1
GAPDH
MCF-7
T47D
SKBR3
MDA-MB-453
MDA-MB-231
MDA-MB-468
Cav-1
Actin

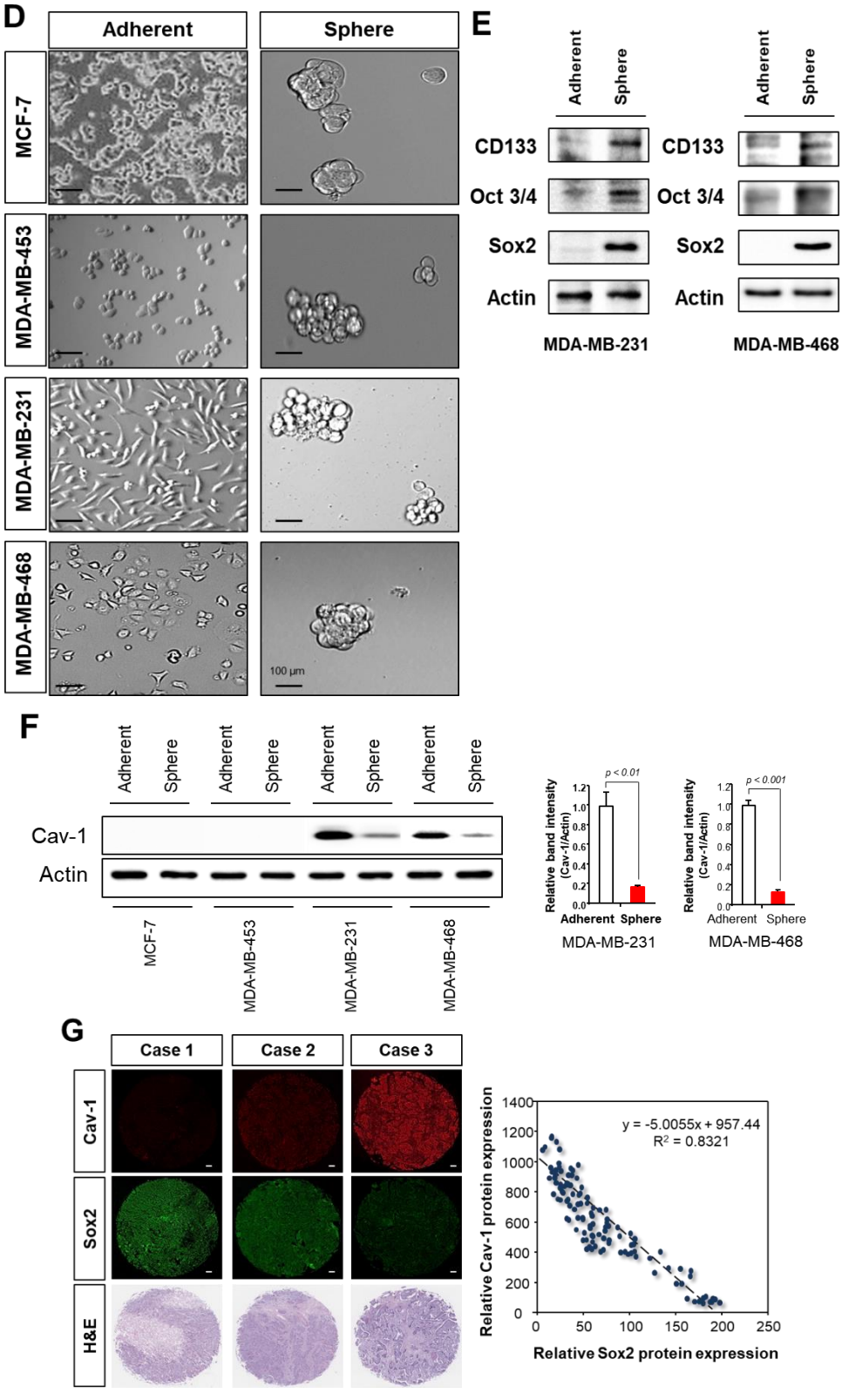

D
Adherent
Sphere
MCF-7
MDA-MB-453
MDA-MB-231
MDA-MB-468
100 μm
E
Adherent
Sphere
CD133
Oct 3/4
Sox2
Actin
MDA-MB-231
MDA-MB-468
F
Cav-1
Actin
MCF-7
MDA-MB-453
MDA-MB-231
MDA-MB-468
p < 0.01
p < 0.001
Relative band intensity (Cav-1/Actin)
Adherent
Sphere
G
Case 1
Case 2
Case 3
Cav-1
Sox2
H&E
y = -5.0055x + 957.44
R² = 0.8321
Relative Cav-1 protein expression
Relative Sox2 protein expression

Figure 2-1. Repression of Cav-1 expression in the spheroids of basal-like breast cancer cells. (A) Immunofluorescent analysis for Cav-1 expression was classified as absent/weak, moderate, or strong in human breast cancer tissues with different clinical stages. The expression of Cav-1 at different levels in each stages was assessed by immunofluorescence staining, and the relative fluorescence intensity was calculated using Image J, an open platform for Java-based scientific image analysis. Hematoxylin and Eosin (H&E) images were provided by US Biomax Inc. (Rockville, MD, USA). Scale bar = 200 μm. **(B)** The expression level of Cav-1 in human breast cancer tissues grouped by invasive stage was assessed by Oncomine database analysis. **(C)** The mRNA and protein levels of Cav-1 in various subtypes of breast cancer cells were measured by reverse transcription-PCR and Western blot analyses, respectively. **(D)** Tertiary tumorspheres derived from breast cancer cells (MCF-7, MDA-MB-453, MDA-MB-231 and MDA-MB-468 cells) were cultured under sphere-forming conditions. For sphere-forming culture, breast cancer cells were exposed to cancer stem cell medium for 24 h. Cells were cultured in an ultra-low attachment plate to generate primary tumorspheres. After 5 days, primary tumorspheres were counted, collected and replated under the same conditions to form secondary tumorspheres. Using the same experimental method, tertiary mammospheres were generated from secondary mammospheres. The number of

mammospheres formed (> 100 μm) was counted under a microscope. Scale bar = 100 μm. **(E)** Expression of stem cell-related marker proteins was analyzed by Western blotting in adherent and sphere cells. **(F)** The expression level of Cav-1 was measured by Western blotting in adherent and sphere cells. The values are expressed as means ± SD (n = 3). **(G)** Immunofluorescent analysis was performed for measuring Cav-1 and Sox2 expression in human breast cancer tissues. The scatter plot demonstrates correlation between Cav-1 and Sox2. Scale bar = 200 μm.

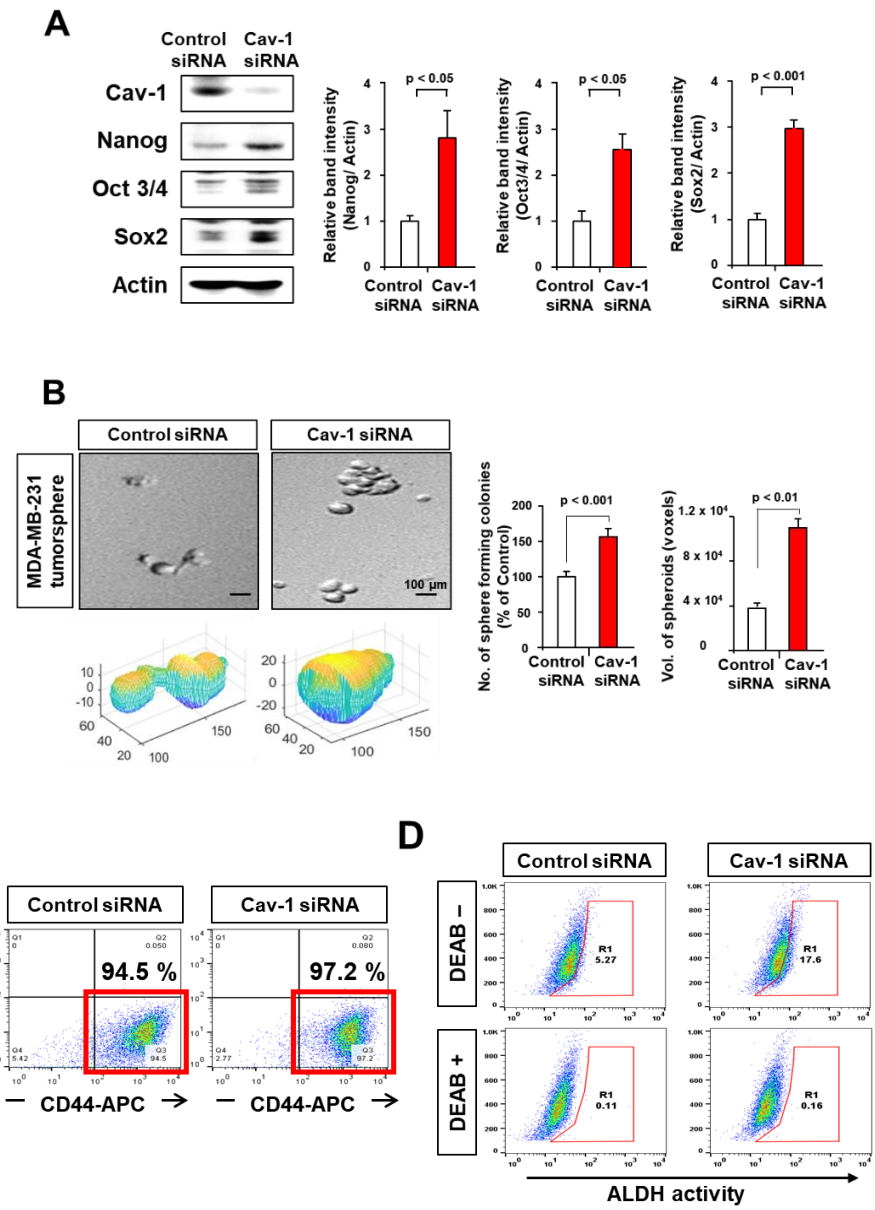
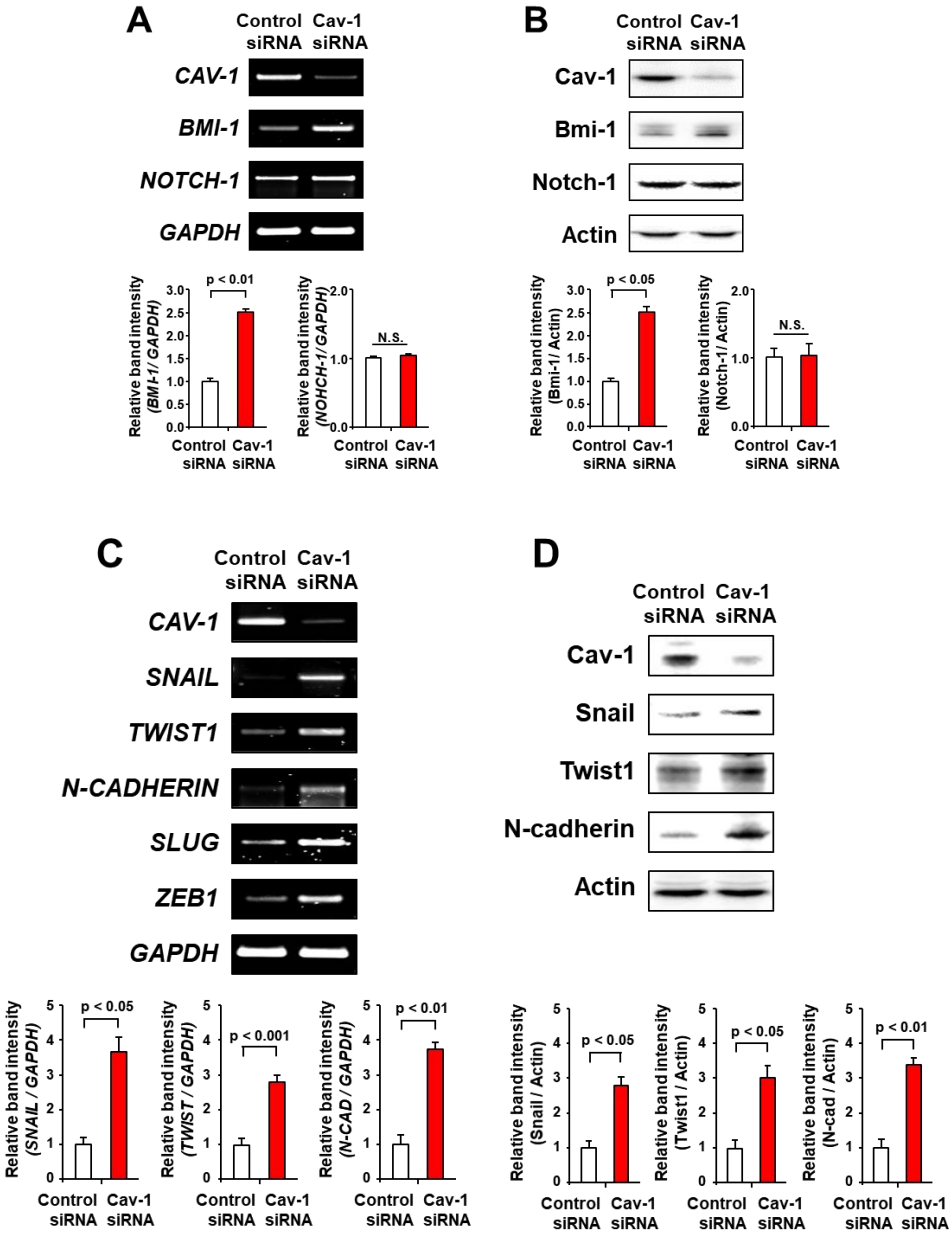


Figure 2-2. Enhancement of the stemness of MBA-MB-231 by Cav-1 knockdown. (**A**) The protein levels of Nanog, Oct 3/4 and SOX2 were assessed by Western blot analysis in the MDA-MB-231 cells transfected with control siRNA or specific Cav-1 siRNA for 36 h. The values are expressed as means ± SD (n = 3). (**B**) After control siRNA or Cav-1 siRNA treatment, MDA-MB-231 cells were cultured

under sphere-forming condition. Tumorsphere frequencies of Cav-1 knockdown MDA-MB-231 cells were calculated and representative sphere images were visualized under a microscope. The volume of each spheroid was computed by using ReViSP, a software specifically designed to accurately estimate the volume of spheroids and to render an image of their 3D surface. The values are expressed as means ± SD (n = 3). **(C)** Proportions of $CD44^{high}$ and $CD24^{low}$ cells were determined by flow cytometry in the MDA-MB 231 cells transfected with control siRNA or specific Cav-1 siRNA for 36 h. The cells were stained with anti-CD44-APC and anti-CD24-PE antibodies. **(D)** Proportions of $ALDH^{+}$ MDA-MB-231 cells were determined by flow cytometry. After cells were treated with control siRNA or specific Cav-1 siRNA for 36 h, the proportions of $ALDH^{+}$ cells were determined by FACS analysis. Lower part: negative control obtained by treating cells with DEAB, an irreversible inhibitor or ALDH activity. Levels of a specific fluorescence were set as background. Upper part: specific ALDH-dependent fluorescence levels in control siRNA or Cav-1 siRNA treated cells. y-axis: side scatter distribution of the tested cell populations. x-axis: distribution of fluorescent cells in the tested cell populations. The values are expressed as means ± SD (n = 3).


A
Control siRNA
Cav-1 siRNA
CAV-1
BMI-1
NOTCH-1
GAPDH
Relative band intensity (BMI-1/ GAPDH)
p < 0.01
Relative band intensity (NOHCH-1/GAPDH)
N.S.
B
Cav-1
Bmi-1
Notch-1
Actin
Relative band intensity (Bmi-1/ Actin)
p < 0.05
Relative band intensity (Notch-1/ Actin)
N.S.
C
CAV-1
SNAIL
TWIST1
N-CADHERIN
SLUG
ZEB1
GAPDH
Relative band intensity (SNAIL / GAPDH)
p < 0.05
Relative band intensity (TWIST / GAPDH)
p < 0.001
Relative band intensity (N-CAD / GAPDH)
p < 0.01
D
Cav-1
Snail
Twist1
N-cadherin
Actin
Relative band intensity (Snail / Actin)
p < 0.05
Relative band intensity (Twist1 / Actin)
p < 0.05
Relative band intensity (N-cad / Actin)
p < 0.01

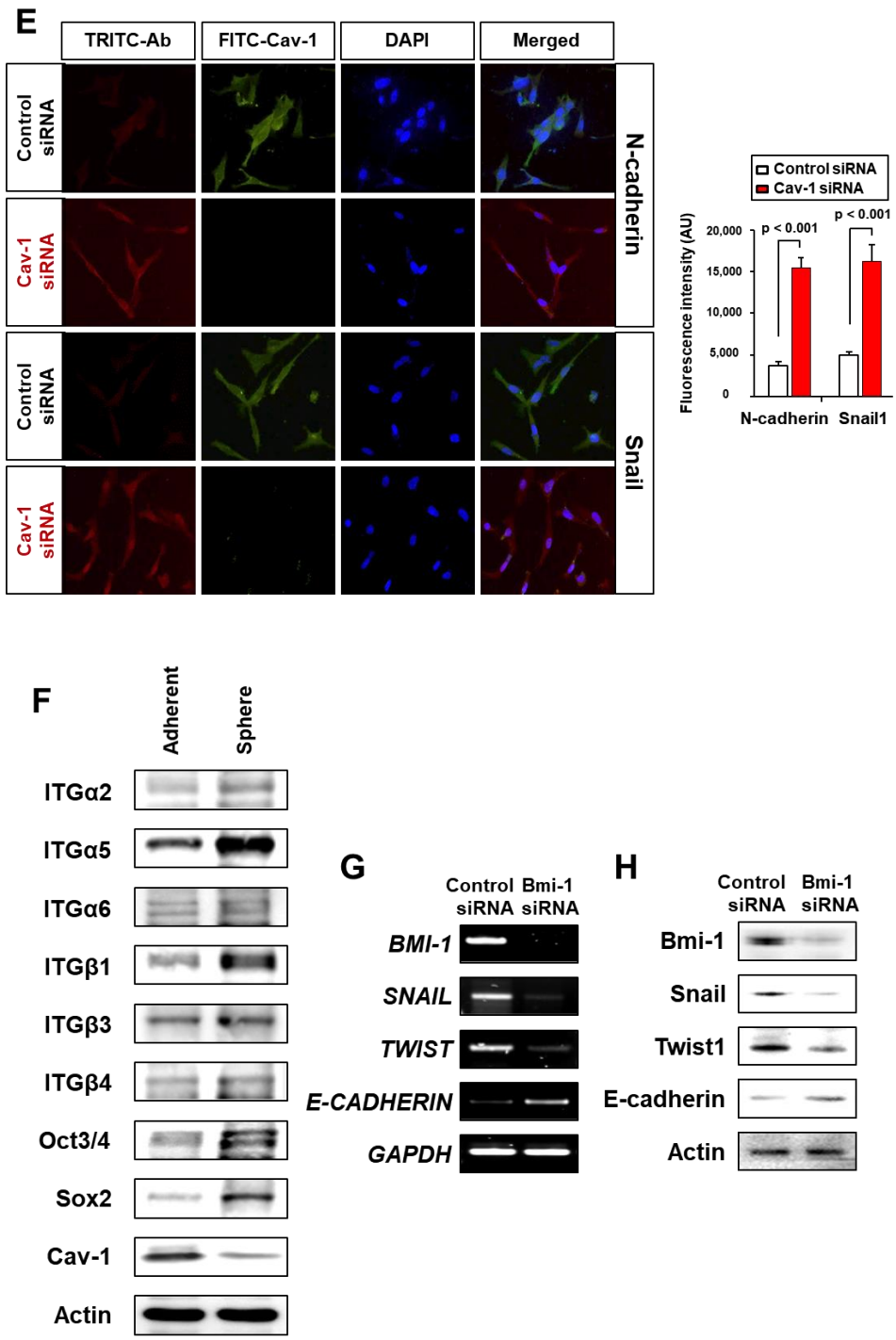

E
TRITC-Ab
FITC-Cav-1
DAPI
Merged
Control siRNA
Cav-1 siRNA
Control siRNA
Cav-1 siRNA
N-cadherin
Snail
Control siRNA
Cav-1 siRNA
Fluorescence intensity (AU)
20,000
15,000
10,000
5,000
0
p < 0.001
p < 0.001
N-cadherin
Snail1
F
Adherent
Sphere
ITGα2
ITGα5
ITGα6
ITGβ1
ITGβ3
ITGβ4
Oct3/4
Sox2
Cav-1
Actin
G
Control siRNA
Bmi-1 siRNA
BMI-1
SNAIL
TWIST
E-CADHERIN
GAPDH
H
Control siRNA
Bmi-1 siRNA
Bmi-1
Snail
Twist1
E-cadherin
Actin

Figure 2-3. Up-regulation of Bmi-1 and EMT markers in Cav-1 knockdown MDA-MB-231 cells. MDA-MB-231 cells were transfected with control siRNA or specific Cav-1 siRNA for 36 h. (**A, B**) RT-PCR and Western blot analysis were performed to detect expression of self-renewal marker genes, *Bmi-1* and *Notch-1* and their protein products, respectively. N.S.; non-significant. (**C, D**) The mRNA and protein levels of EMT markers were determined by RT-PCR and Western blot analysis, respectively. **(E)** Immunocytochemical analysis was performed using antibodies against N-cadherin and Snail. Conditions for Cav-1 knockdown MDA-MB-231 cells are same as described in (**A**). Cells stained with DAPI were visualized by fluorescent microscopy. Relative mean fluorescence intensity was calculated using Image J. **(F)** The expression level of integrins were measured by Western blotting in adherent and sphere cells. (**G, H**) RT-PCR and Western blot analysis of EMT markers in MDA-MB-231 cells that were transfected with non-specific or Bmi-1 specific siRNA for 36 h. GAPDH and β-actin were used as a loading control.

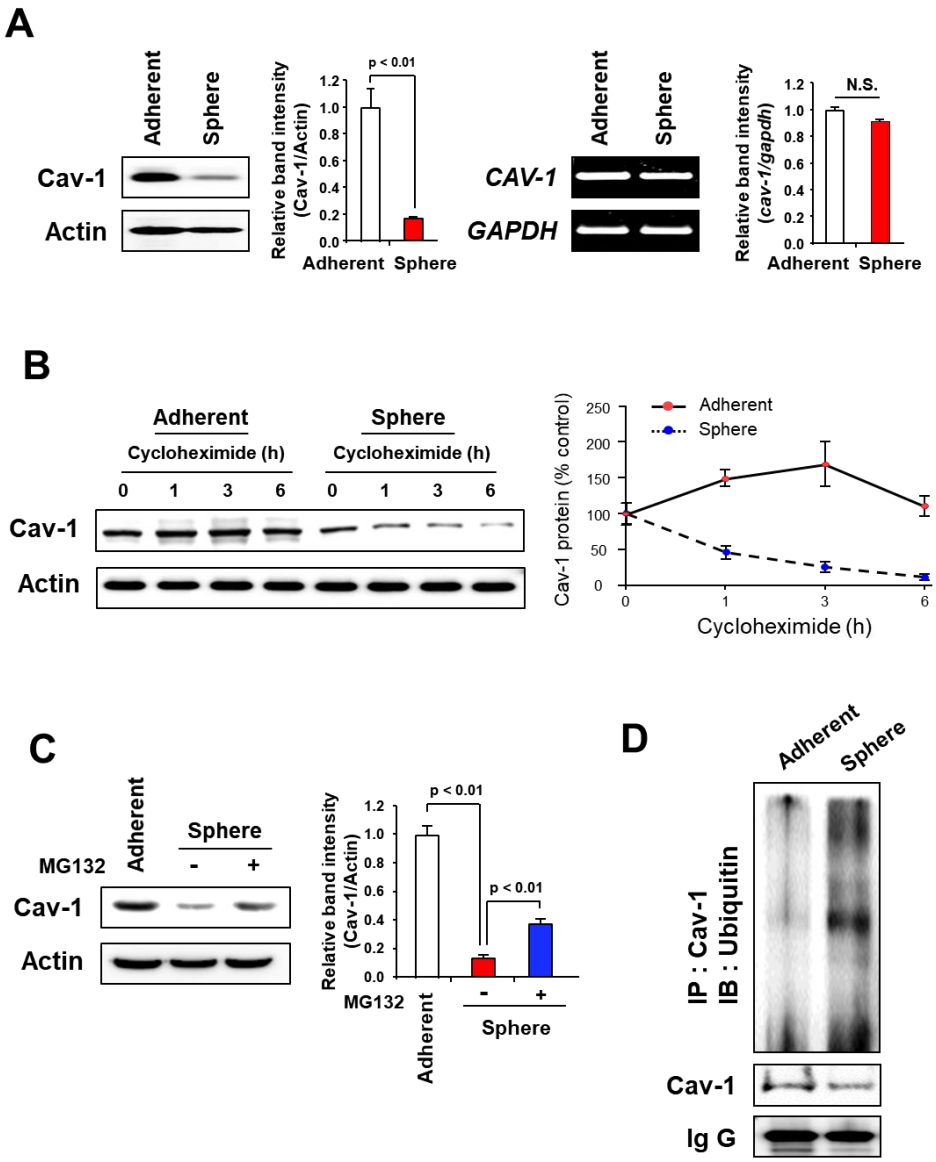


Figure 2-4. Enhanced ubiquitination and proteasomal degradation of Cav-1 protein in MDA-MB-231 tumorspheres. (**A**) MDA-MB-231 cells were cultured on ultra-low attachment plates for 5 days to generate primary tumorspheres, and then were dissociated into single cell suspension to generate secondary tumorspheres for 5 days. Using the same experimental method, tertiary tumorspheres were generated

from secondary tumorspheres. The expression of Cav-1 and its mRNA transcript was determined by Western blot and RT-PCR analyses, respectively in adherent and MDA-MB-231 tumorspheres. β-Actin and *GAPDH* were used as a loading or internal control. N.S.; non-significant. **(B)** To monitor the protein stability, adherent and MDA-MB-231 tumorspheres were treated with 70.07 μM of cycloheximide (CHX), and the expression levels of Cav-1 was measured at indicated time points. **(C)** Tertiary tumorsphere cells were treated with or without 20 μM of MG-132, and expression levels of Cav-1 were determined by Western blot analysis as compared to adherent cells. The values are presented means ± SD (n = 3). **(D)** To assess the ubiquitination of Cav-1, adherent and MDA-MB-231 tumorspheres were immunoprecipitated with anti-Cav-1 antibody, followed by Western blot analysis with anti-ubiquitin antibody.

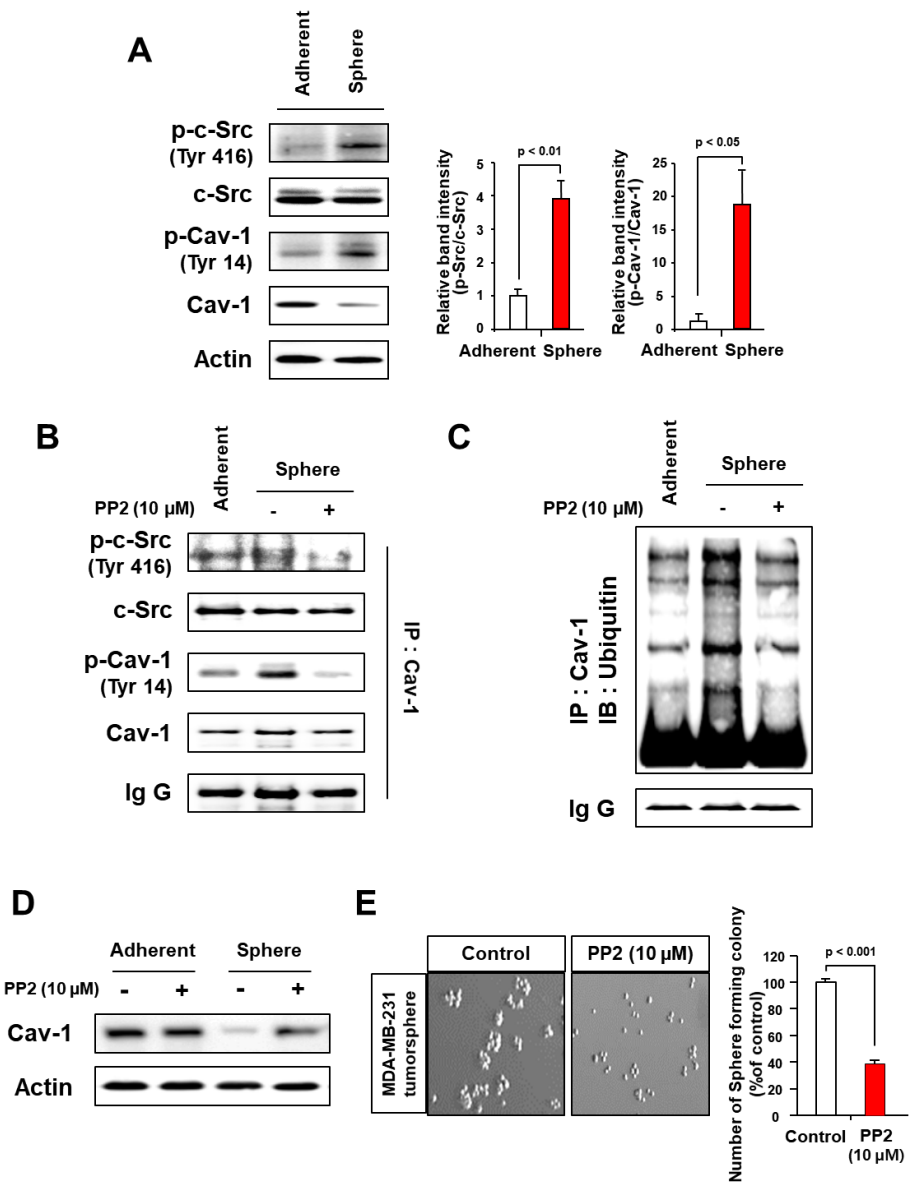


Figure 2-5. Effect of Src on phosphorylation and stability of Cav-1 in MDA-MB-231 tumorspheres. (A) The expression levels of total and Tyr416 phosphorylated Src and Cav-1 phosphorylated at Tyr14 were measured by Western blot analysis in adherent and MDA-MB-231 tumorspheres. The values are presented means ± SD (n = 3). **(B)** Tertiary tumorspheres were treated with PP2 (10 μM) or

DMSO for 24 h before the sample collection. Src and Cav-1 phosphorylated at Tyr416 and Tyr14, respectively as well as their total forms in adherent and MDA-MB-231 tumorspheres were measured by immunoprecipitation with anti-Cav-1 antibody, followed by immunoblot analysis with antibodies against corresponding antibodies. **(C)** The ubiquitination of Cav-1 was measured by immunoprecipitation as described in the legend to Fig. 2-4D. **(D)** Following treatment of adherent and tertiary tumorsphere cells with PP2 (10 μM) or DMSO, the protein level of Cav-1 was examined by Western blot analysis. **(E)** The effects of PP2 on tumorsphere formation was confirmed by the tumorsphere assay. Alterations in the shape of tumorspheres were examined by phase-contrast microscopy. The histogram represents the number of spheres that were bigger than 100 μm. The values are indicated as means ± S.D. (n = 3).

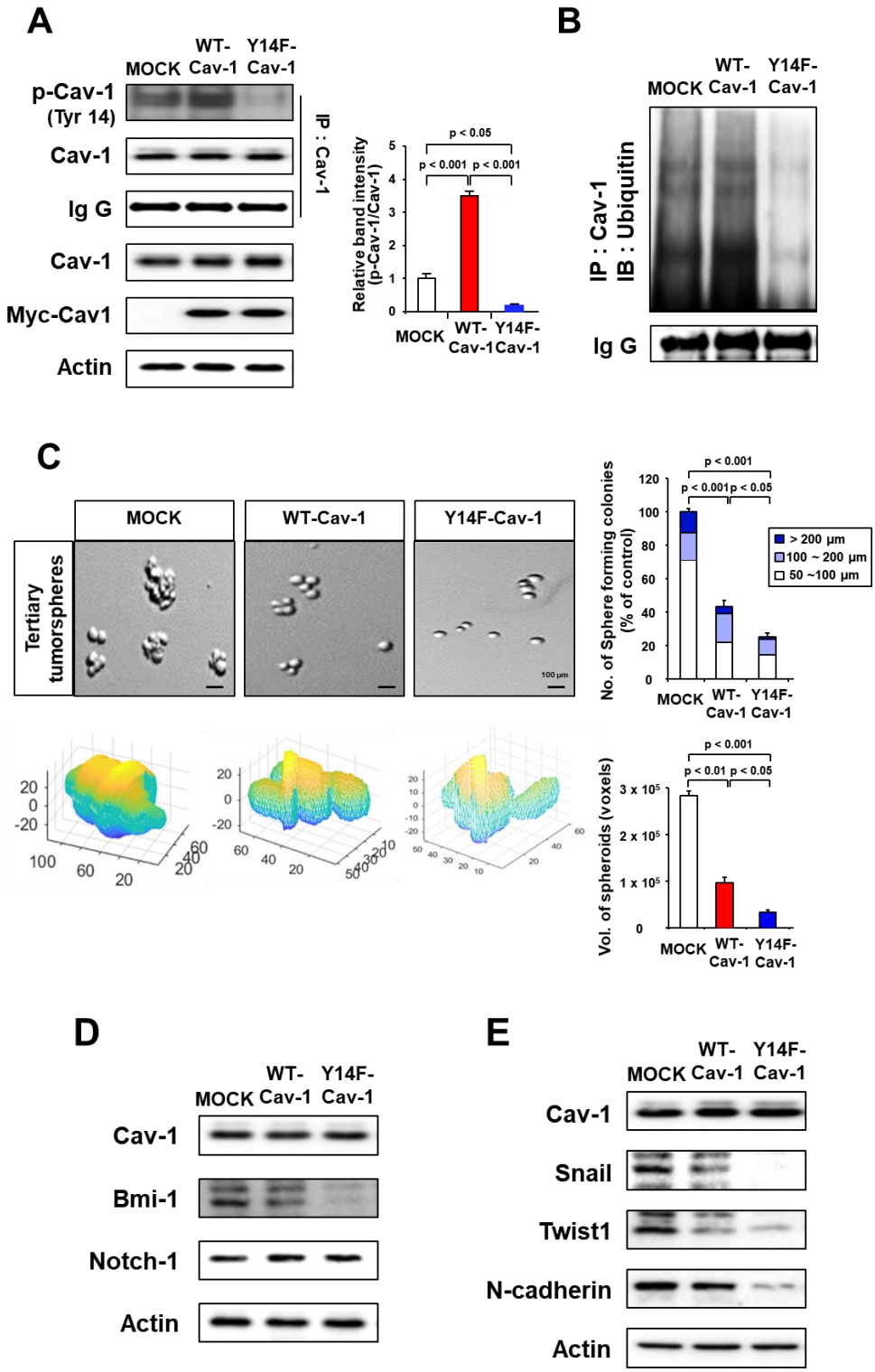
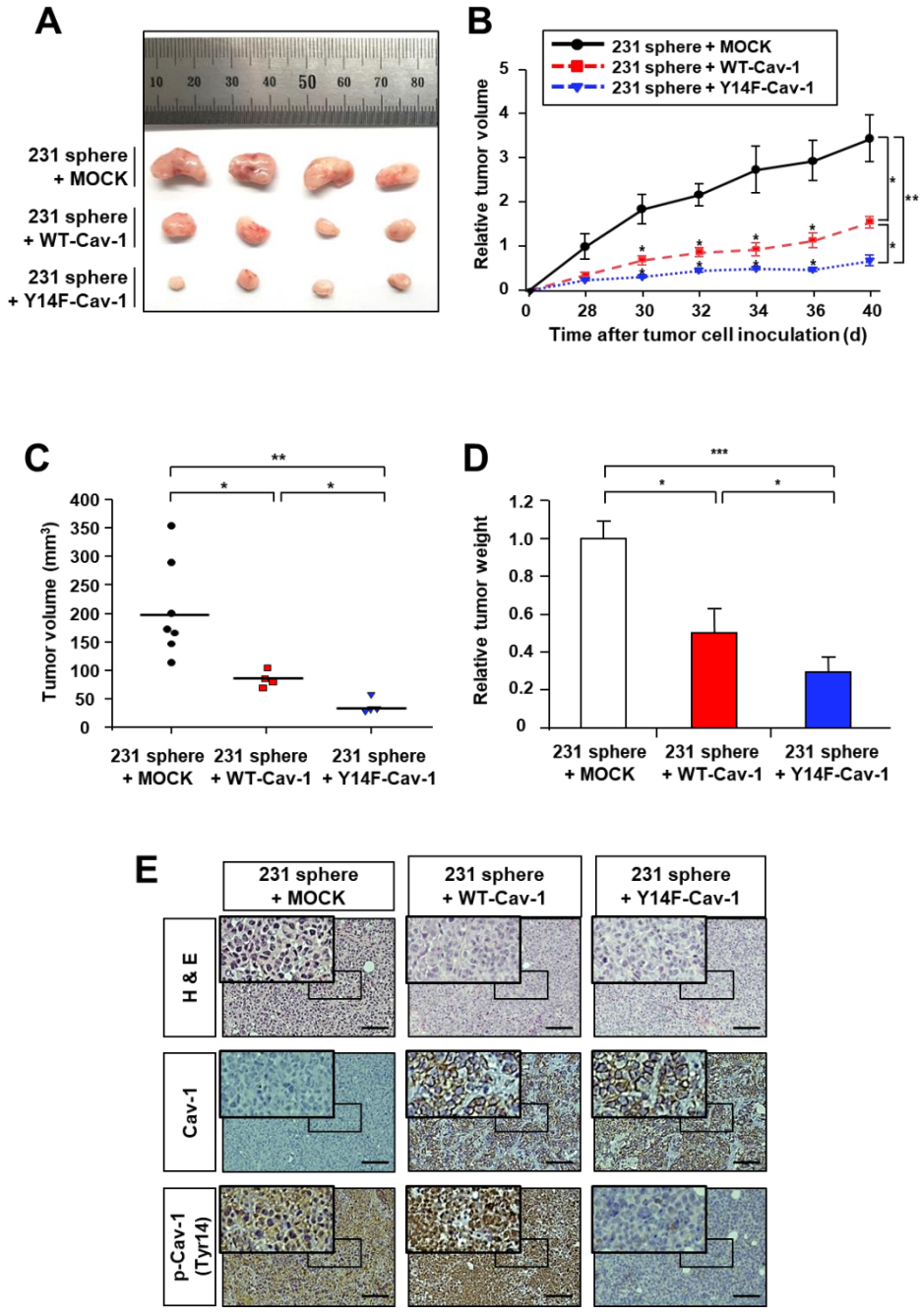

A
MOCK
WT-Cav-1
Y14F-Cav-1
p-Cav-1 (Tyr 14)
Cav-1
Ig G
Cav-1
Myc-Cav1
Actin
IP : Cav-1
Relative band intensity (p-Cav-1/Cav-1)
p < 0.05
p < 0.001
p < 0.001
B
IP : Cav-1
IB : Ubiquitin
Ig G
C
MOCK
WT-Cav-1
Y14F-Cav-1
Tertiary tumorspheres
100 μm
No. of Sphere forming colonies (% of control)
> 200 μm
100 ~ 200 μm
50 ~100 μm
Vol. of spheroids (voxels)
p < 0.01
D
Cav-1
Bmi-1
Notch-1
Actin
E
Cav-1
Snail
Twist1
N-cadherin
Actin

Figure 2-6. Essential role of tyrosine 14 of Cav-1 in maintaining stemness of breast cancer cells *in vitro*. MDA-MB-231 cells were transiently transfected with Myc-tagged wild-type (WT-Cav-1) or mutant-Cav1 (Y14F-Cav-1). **(A)** The tyrosine phosphorylation of Cav-1 was detected by immunoprecipitation followed by immunoblot analysis. The expression levels of Cav-1 and myc-tagged Cav-1 in clones expressing MOCK (vector alone) or WT-Cav-1/Y14F-Cav-1 (vector containing myc-tagged Cav-1) were measured by Western blot analysis. **(B)** The ubiquitination of Cav-1 was measured by immunoprecipitation. **(C)** Tumorsphere-forming ability was calculated, and representative sphere images were visualized under a microscope. The volume of each spheroid was computed by using ReViSP, a software specifically designed to accurately estimate the volume of spheroids and to render an image of their 3D surface. The values are expressed as means ± SD (n = 3). The expression level of self-renewal **(D)** and EMT markers **(E)** were analyzed by Western blotting.


A
231 sphere + MOCK
231 sphere + WT-Cav-1
231 sphere + Y14F-Cav-1
B
231 sphere + MOCK
231 sphere + WT-Cav-1
231 sphere + Y14F-Cav-1
Relative tumor volume
Time after tumor cell inoculation (d)
C
Tumor volume (mm3)
231 sphere + MOCK
231 sphere + WT-Cav-1
231 sphere + Y14F-Cav-1
D
Relative tumor weight
231 sphere + MOCK
231 sphere + WT-Cav-1
231 sphere + Y14F-Cav-1
E
231 sphere + MOCK
231 sphere + WT-Cav-1
231 sphere + Y14F-Cav-1
H & E
Cav-1
p-Cav-1 (Tyr14)

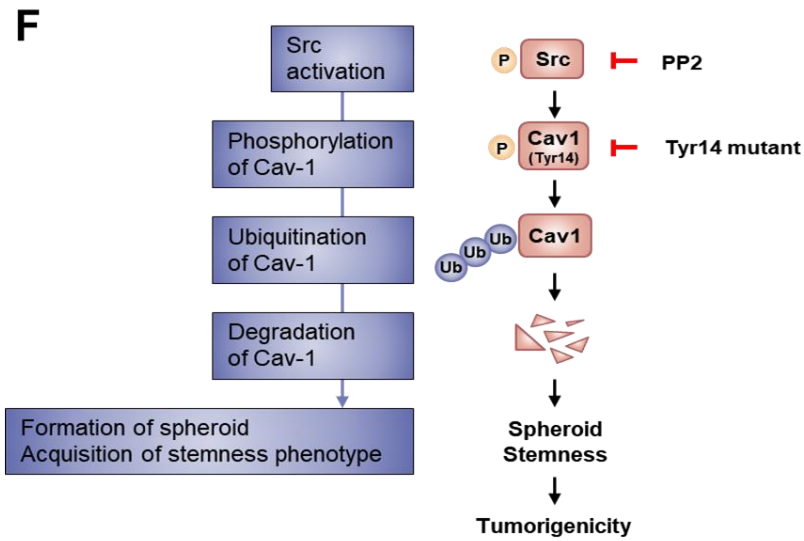


Figure 2-7. A critical role of tyrosine 14 of Cav-1 in growth and proliferation of MDA-MB-231 tumorspheres transplanted to nude mice. MDA-MB-231 cells transfected with mock or Myc-tagged wild-type (WT-Cav-1) or mutant-Cav1 (Y14F-Cav-1) were cultured in cultured in ultra-low attachment plates and then passaged to tertiary tumorspheres as described in Materials and methods. A total 1×10^4 cells were injected into the mammary fat pad of BALB/c nude mice. **(A)** Representative tumor images excised from mice at the end of the experiment at the day 40. **(B)** The tumor volume was measured by using the following formula; Volume (V) = 0.5 × longest diameter × (shortest diameter)^2. The values are indicated as means ± S.D. of six xenografts for each group, *$p < 0.05$, **$p < 0.01$. **(C)** Histograms of the mean tumor volume of each group. Mean tumor volume for each group was calculated at 40 days after injection. **(D)** The tumor weight was measured at the end of the

experiment. The results are expressed as means ± SD., *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (**E**) Immunohistochemical stains of Cav-1 and p-Cav-1 in tumors from mice. Scale bar = 200 μm.

5. Discussion

Chemo-resistance and disease relapse in cancer are attributed to a small subset of cancer stem cells (CSCs) with the capability of self-renewal and differentiation [33, 34]. Several studies have demonstrated association between stemness and the metastatic potential of disseminated tumor cells [28, 35-37]. In the tumor progression, disseminated cancer cells may display a more mesenchymal phenotype, bestowing these cells with stem-like traits. The transformed cells with stem-like traits can migrate from the primary tumor to the bone marrow, due to their capacity to perform the EMT. CSCs localize in the pre-metastatic niche, a distinct region responsible for metastatic progression [38-41]. According to the model of CSCs, a small subpopulation of cancer cells is endowed with stem like traits with the potential to promote cancer progression [42]. CSCs are also primarily responsible for the recurrence of cancer. Therefore, eradication of CSCs in tumors may represent an effective anticancer therapeutic strategy. So far, significant efforts have been made to explore the signaling pathways modulating self-renewal and differentiation of CSCs, to develop regimens or therapeutic strategies targeting CSCs [43, 44].

Several lines of evidence suggest that Cav-1 may function as a regulator of self-renewal signaling pathways in stem cells [45, 46]. In various cancer types, Cav-1 levels vary during the course of tumor progression. It has been speculated that Cav-

1 acts both as a tumor suppressor and an oncogene, depending on the stage of neoplastic transformation and extent of tumor progression [47, 48]. Cav-1 is down-regulated in early stages facilitating oncogenic transformation, while restoration of Cav-1 in later stages possibly contributes to the development of invasiveness and drug resistance. However, the precise function of Cav-1 on acquisition of stem cell-like properties in cancer progression is largely unknown.

The tumorsphere culture system has been widely used to identify and enrich for putative CSCs from cancer cell lines or primary tumors [49]. In this study, four human breast cancer cell lines representing the major molecular subtypes of breast cancer (Luminal; MCF-7, HER2; MDA-MB-453, Basal-like; MDA-MB-231 and MDA-MB-468) were exploited to generate tumorspheres. I observed that tumorspheres derived from breast cancer cells exhibited much lower levels of Cav-1, compared with adherent cells. Silencing of Cav-1 with siRNA induced stemness properties as evidenced by increased $CD44^{high}/CD24^{low}$ cell population, ALDH activity, expression of stemness-related genes and tumorsphere formation. Recently, the $CD44^{high}/CD24^{low}$ and $ALDH^{+}$ phenotypes are less frequently utilized to identify of CSCs, because their expression is not consistent even in the same molecular subtype of breast cancer. Systematic comparison of their functions is also still insufficient [50, 51]. Nevertheless, their expression is still used to identify breast

CSCs. Another frequently used stem cell marker is Oct4 (octamer-binding transcription factor 4). According to a hierarchy of breast cancer cells proposed by Patel et al. [52], there is a subset of cells with the least maturity that express a high level of Oct4. This most immature subset of $Oct4^{high}$ breast cancer cells demonstrates chemoresistance, dormancy, and stem cell properties, such as self-renewal, serial passaging ability, cycling quiescence, long doubling time, asymmetric division, high metastatic and invasive capability.

Several studies highlighted the role of cadherins and integrins, not only in the regulation of EMT but also in maintaining CSC [53-55]. Consistent with these findings, I noticed that the expression of α5β1 integrin was increased in tumorspheres. Furthermore, Cav-1 expression has been found to be significantly associated with α5β1 integrin [56]. Results from our present study suggest that Cav-1 knockdown led to EMT which endows breast cancer cells with stem-like features. Similar effects of Cav-1 on stemness were also observed in other cancer cell lines [57], further confirming that Cav-1 plays a role in the cancer progression.

Decreased protein expression of Cav-1 in tumorspheres is not attributable to reduced expression of its mRNA transcript, but rather due to lowered protein stability. It has been reported that phosphorylation of Cav-1 at tyrosine 14 reduces Cav-1

protein stability by facilitating the ubiquitination and proteasomal degradation [58]. Cav-1 is mainly phosphorylated on tyrosine 14 by Src kinase [15, 19, 59]. Phosphorylation on Tyr 14 by Src is clearly relevant to Cav-1 functions in a number of settings, such as EGF-induced caveolae formation [60], integrin-regulated membrane microdomain internalization [61], and association with the membrane type-I matrix metalloproteinase [19]. Our finding reveals that the tyrosine residue at position 14 of Cav-1 is required for its modulating spheroid formation, and acquiring EMT and stem-like traits. Cav-1 mutation by replacing the Tyr14 with phenylalanine significantly impaired tumorigenic ability of tumorspheres derived from human breast cancer cells.

In summary, the down-regulation of Cav-1 is detected in breast CSC-like cells, which is associated with upregulation of Bmi-1 and EMT markers. Cav-1 negatively regulates CSC markers, including CD133, Oct 3/4, and Sox2 and tumor spheroid formation, a key feature of CSCs. Down-regulation of Cav-1 is attributed to its destabilization through the Src-mediated phosphorylation at the Tyr14 and subsequent degradation via the ubiquitin-proteasome pathway. Cav-1 destabilized cells exhibit stem-like characteristics which facilitate the formation of tumorspheres and enhance the tumorigenicity (**Fig. 7F**). Together, our results unveil a novel mechanism of CSC regulation, which could be crucial in understanding the

aggressive behaviors of cancer cells and in identifying potential targets for CSC therapy.

6. References

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Chapter III

Caveolin-1 inhibits stem-like traits of breast cancer cells through direct interaction with Nrf2

1. Abstract

Breast cancer is a fatal tumor and comprises heterogeneous cells in which a sub-population with stem cell-like properties is included. Cancer cells with stem cell-like traits account for tumor progression, metastasis and recurrence. Therefore, identification and characterization of specific factors regulating stem-like traits are critical for development of breast cancer therapeutics. In the present study, Caveolin-1 (Cav-1), the plasma membrane component protein, was found to regulate stem-like traits of breast cancer cells through direct interaction with NF-E2-related factor 2 (Nrf2). Biochemical studies show that Nrf2 colocalized with Cav-1, and Cav-1 limited the migration of Nrf2 to the nucleus in adherent cells. In contrast, Nrf2 was constitutively localized in the nucleus of tumorspheres, which exhibited low expression of Cav-1. Functional studies demonstrated that knockdown of Cav-1 exhibited the increased stemness signaling molecules and EMT markers, which was decreased by Nrf2 siRNA treatment. Taken together, Cav-1 interacts with Nrf2 in breast cancer and inhibits Nrf2 nuclear translocation, thereby suppressing CSC-like properties.

Keywords: breast cancer stemness; Caveolin-1; Nrf2; direct interaction; nuclear translocation

2. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death in women worldwide [1]. Accumulating evidence suggests that a small subpopulation of tumor cells, called cancer stem cells (CSCs) are implicated in tumor progression, recurrence and therapeutic resistance [2, 3]. However, the molecular mechanisms underlying manifestation and maintenance of stem-like properties remain largely unknown.

Caveolin-1 (Cav-1) is a scaffolding protein primarily located in the lipid raft domains of the cellular plasma membrane. Cav-1 participate in various cellular functions, such as cellular metabolism, endocytosis, vesicle trafficking, cholesterol homeostasis, tumor progression and signal transduction Cav-1 has been known to interact with various important molecules such as nitric oxide synthase, epidermal growth factor receptor, Src-like nonreceptor tyrosine kinases, and protein kinase A [4, 5]. It has been reported that Cav-1 promotes the cancer stem cell-regulatory activity [6].

Nuclear erythroid 2 p45-related factor-2 (Nrf2) is a key transcription factor that regulates the antioxidant gene expression [7]. Under homeostatic conditions, Keap1 (Kelch-like ECH-associated protein 1) binds to Nrf2 and facilitates the degradation

of Nrf2 via the proteasome system [8]. Upon stimulation, Nrf2 dissociates from its cytoplasmic inhibitor Keap1, translocates to the nucleus, and transactivates the expression of antioxidant genes [9, 10]. Although Nrf2 exhibits a beneficial effects in normal cells, the constitutive overactivation of this transcription factor has been observed in some tumors [11]. Overactivation of Nrf2 also confers chemoresistance in cancer cells through upregulation of antioxidant enzymes [12]. Recent studies have demonstrated that Nrf2 signaling is involved in CSC-like properties of several types of malignancies [13-15]. However, how Cav-1 regulates the expression of Nrf2 in cancer stem cells remains unclear. Our previous studies have demonstrated that Nrf2 and its target protein, HO-1 augment stem-like traits of breast cancer cells [16]. This prompted us to further investigate whether Cav-1 could regulate stemness by targeting the Nrf2-HO-1 axis. It has been reported that Cav-1 physically interacts with Nrf2, thereby suppressing its antioxidant activity in normal cells [17, 18]. In the present study, I found that Cav-1 could interact with Nrf2 in breast cancer and inhibit the Nrf2 signaling, thereby suppressing the manifestation of CSC-like properties.

3. Materials and methods

Reagent and antibodies

Dulbecco's modified Eagle's medium (DMEM), Rosewell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). TRIzol® was obtained from Invitrogen (Carlsbad, CA, USA). Primary antibodies for Bmi-1, ubiquitin and α-tubulin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Cav-1, Nrf2, Snail, Lamin B and β-actin were obtained from Santa Cruz (Santa Cruz, CA, USA). Antibody against N-cadherin was purchased from BD Biosciences (Bedford, MA, USA). The bicinchoninic acid (BCA) protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL, USA).

Cell culture

The human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-468 were obtained from the Korean Cell Line Bank (Seoul, South Korea). MDA-MB-231 and MDA-MB-468 cells were maintained in DMEM, and MCF-7 cells were maintained in RPMI 1640 culture medium. All culture media were supplemented with 10% FBS and 1% antibiotics and cells were grown at 37℃ with 5% CO_2 / 95 % air.

Tumorsphere culture

For tumorsphere formation from the adherent cells, single cells were cultured in a serum free DMEM/F12 medium supplemented with B27 (GIBCO), 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL basic fibroblast growth factor (b-FGF; PeproTech, Rocky Hill, NJ, USA) and 4 ng/mL heparin (Sigma-Aldrich). Primary tumorspheres were seeded at a density of 1 x 10^4 cells/mL in 100 mm ultralow attachment plates (Corning, NY, USA) for 5 consecutive days, and 2 mL of medium was added every third day. To culture secondary tumorspheres, primary tumorspheres were gently collected and dissociated into a single-cell suspension using 40 μm strainer. Single cells were counted and then seeded again for another 5 days with addition of 2 mL medium every third day. Using the same experimental method, tertiary mammospheres were generated from secondary mammospheres. The number of mammospheres formed (> 100 μm) was counted at indicated times under a microscope.

Fractionation of cytosolic and nuclear extracts

Cells were washed with ice-cold PBS, scraped in 1 mL PBS and centrifuged at 7,000 *g* for 15 min at 4 ℃. Pellets were suspended in 50 μL of hypotonic buffer A [10mM

HEPES (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitiol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) for 15 min on ice, and 1 μL of 10% Nonidet P-40 solution was added for 5 min. The mixture was then centrifuged at 12,000 *g* for 7 min. The supernatant was collected as a cytosolic fraction. The pellets were washed with hypotonic buffer and were resuspended in hypertonic buffer C [20 mM HEPES (pH 7.8), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2mM PMSF] for 30 min on ice and centrifuged at 12,000 *g* for 7 min. The supernatant containing nuclear proteins was collected and stored at -70 ℃ after determination of the protein concentration by the Bradford method using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis.

Whole cell lysate was prepared by scapping the cells in RIPA lysis buffer [150 mM NaCl, 0.5% Triton x 100, 50 mM Tri-HCl (pH 7.4), 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 0.1 mM PMSF] for 15 min on ice followed by centrifugation at 13000 *g* for 15 min. The supernatant containing proteins was collected and stored at -70℃. For Western blot analysis, the protein concentration of whole cell lysates was measured by using the BCA protein assay kit (Pierce, Rockford, IL, USA). Lysates from cells were separated by running through 8-12%

sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred to the polyvinylidene difluoride (PVDF) membranes (Gelman Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5% non-fat dry milk/TBST (Tris-buffered saline buffer containing 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated with the respective primary antibody diluted in TBST overnight 4℃. Blots were rinsed three times with TBST at 10-min intervals followed by incubation with respective horseradish peroxidase conjugated secondary antibodies (rabbit, mouse or goat) in TBST for 1 h at room temperature. The blots were washed again three times with TBST. The band images in Western blotting were visualized with an enhanced chemiluminescent (ECL) detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and quantified with the LAS-4000 Image Analyzer (Fujifilm, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was isolated from each cell by using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To generate the cDNA from RNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) for 50 min at 42°C and again for 15 min at 72°C. About 1 μL of cDNA was amplified with a PCR mixture

(HS Prime Taq 2X Premix, Daejeon, South Korea) in sequential reactions. The primers used for each RT-PCR reactions are as follows : *CAV-1*, 5'-ATG TCT GGG GGC AAA TAC GTA-3' and 5'–TTG GAA CTT GAA ATT GGC ACC A-3'; *NRF2*, 5'-ACT GGT TGG GGT CTT CTG TG-3' and 5'-CGG TAT GCA ACA GGA CAT TG-3'; *HO-1*, 5'-TAC ACA TCC AAG CCG AGA AT-3' and 5'-GTT CCT CTG TCA GCA TCA CC-3'; *GAPDH,* 5'-AAG GTC GGA GTC AAC GGA TTT-3' and 5'-GCA GTG AGG GTC TCT CTC T-3' (forward and reverse, respectively). Amplification products were analyzed by 1.5-2% agarose gel electrophoresis, followed by staining with SYBR Green (Invitrogen, Carlsbad, CA, USA) and photographed using fluorescence in LAS-4000 (Fujifilm, Tokyo, Japan).

Transient transfection of siRNA

MDA-MB-231 cells were seeded at a density of 1 x 10^5 cells/mL in 100 mm dish in complete growth media. Cells were transfected with 25 nM of specific or control siRNA oligonucleotides using Lipofectamine RNAiMAX according to manufacturer's instruction (Invitrogen). The sense and antisense strands of Cav-1 and Nrf2 siRNA used are as follows (forward and reverse, respectively): Cav-1; 5' -AGA CGA GCU GAG CGA GAA GCA UU3' (forward) and 5' -UGC UUC UCG CUC AGC UCG UCU UU-3' (reverse) Nrf2; 5'-

AAGAGUAUGAGCUGGAAAAAC-3' and 5'-GUUUUUCCAGCUCAUACUCUU-3'. siRNA oligonucleotides targeting for Cav-1 and Nrf2 were purchased from Genolution Pharmaceuticals (Seoul, South Korea).

Transient transfection of plasmid

Transient transfection of plasmid encoding native Cav-1 was performed by Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 36-h transfection, cells were harvested or cultured to generate according to purpose of the experiment.-

Immunocytochemical analysis

Cells were plated on the 8-well chamber slide (0.5 x cells/well) and fixed in 95% methanol for 10 min at -20℃. After rinse with PBS containing 0.1% Tween 20 (PBST), cells were incubated in 0.2% Triton X-100 in PBS for 5 min. After three washing steps with PBST, cells were blocked for 2 h in fresh blocking buffer [PBST (pH 7.4) containing 5% bovine serum albumin (BSA)]. Dilution (1:100) of primary antibody was made in PBST with 1% BSA, and cells were incubated overnight at 4℃. After three washing steps with PBST, the cells were incubated with a diluted (1:1000) TRITC-conjugated anti-mouse or FITC-conjugated anti-rabbit IgG

secondary antibody in PBST with 1% BSA at room temperature for 1 h. Cells were also stained with 4',6-diamidino-2-phenylindole (DAPI) and rinsed with PBST. Stained cells were analyzed under a confocal microscope (Leica Microsystems, Heidelberg, Germany) and photographed.

Immunoprecipitation

Cells were lysed in 250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 μM NaF, 2 μM sodium orthovanadate, 1 mM PMSF and 10 mM N-ethylmaleimide. Total protein (500 μg) was subjected to immunoprecipitation by shaking with Cav-1 primary antibody at 4℃ for 24 h followed by the addition of 20 μL of 25% protein G-agarose bead slurry (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA) and additional shaking for 2 h at 4℃. After centrifugation at 10,000 *g* for 1 min, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. After final wash, immunoprecipitate was resuspended in 50 μL of 2X SDS electrophoresis sample buffer and boiled for 5 min. Forty five μL of supernatant from each sample was loaded on SDS-PAGE. The ubiquitinated Nrf2 was visualized by antibody against ubiquitin.

Statistical analysis

Data were represented as means of ± standard deviation [10] at least three independent experiments. Statistical significance was determined by Student's *t*-test and a *p*-value of less than 0.05 was considered to be statistically significant.

4. Results

Interactions between Cav-1 and Nrf2

To investigate the relationship between Cav-1 and Nrf2, we used MCF7 and MDA-MB-231 human breast cancer cells, which express relatively low and high levels of both proteins, respectively (**Fig. 3-1A**). Immunoprecipitation analysis demonstrated that siRNA-dependent knockdown of Cav-1 decreased the Cav-1 and Nrf2 interaction in MDA-MB-231 cells (**Fig. 3-1B**, right panels), whereas co-overexpression of Cav-1 and Nrf2 eventually increased their interactions in MCF-7 cells (**Fig. 3-1B**, left panels). To further explore the association between Cav-1 and Nrf2 could be involved in the stemness of breast cancer cells, I enriched for stemness characteristics of breast cancer cells, by culturing MDA-MB-231 cells as spheroids. Tumorspheres derived from MDA-MB-231 breast cancer cells exhibited much lower levels of Cav-1 with concomitant increases in Nrf2 accumulation, compared with adherent cells (**Fig. 3-1C, left panels**). Further, the interaction between Cav-1 and Nrf2 was found to be decreased in tumorspheres (**Fig. 3-1C, right panels).**

Regulation of Nrf2 activity by Cav-1

To explore whether the interaction between Cav-1 and Nrf2 affects Nrf2 signaling, endogenous Cav-1 expression was blocked by small interfering RNA (siRNA) in MDA-MB-231 cells. We found that Cav-1 knockdown increased the expression of Nrf2 and HO-1 as well as their mRNA transcripts (**Fig. 3-2A**). Consistent with these findings, overexpression of Cav-1 inhibited the expression of Nrf2 and HO-1 in MCF-7 cells at both transcriptional and translational levels (**Fig. 3-2B**). These data, taken all together, suggest that Cav-1 inhibits Nrf2 signaling in breast cancer through direct interaction with this transcription factor.

Cav-1 inhibits translocation of Nrf2 to the nucleus

In another experiment, the association between Cav-1 and Nrf2 was investigated in subcellular fractions. As shown in **Fig. 3-3A** and **3B**, Nrf2 was significantly accumulated in the nucleus of MDA-MB-231 cells after treatment with Cav-1 siRNA. Nrf2 is degraded by the 26S proteasome system after multiple ubiquitylation [19]. Silencing of Cav-1 caused a decrease in Nrf2 ubiquitination in MDA-MB-231 cells (**Fig. 3-3C**). Immunofluorescence studies demonstrated Nrf2 enriched predominantly in the nucleus of tumorspheres, which have low expression of Cav-1 (**Fig. 3-4A**). In contrast, Cav-1 overexpression in MCF-7 tumorspheres,

abolished nuclear accumulation of Nrf2 in the nucleus (**Fig. 3-4B**). Out results indicate that Cav-1 inhibits the translocation of Nrf2 to the nucleus, which might facilitate the ubiquitination of Nrf2.

Nrf2 suppressed expression of Bmi-1, stem-like and EMT markers induced by Cav-1

Self-renewal is one of the representative characteristics of CSCs which accounts for the regeneration of the tumor [20]. Bmi-1 is the critical proteins involved in regulation of self-renewal signaling [21, 22], and Oct3/4 and Sox2 regulate self-renewal and tumorigenicity of stem-like cells [23, 24]. Moreover, EMT is essential for generation of cancer stem cells [25, 26]. In our previous study, loss of Cav-1 promotes breast cancer stemness by acquiring EMT and stem-like traits. The underlying molecular mechanisms by which Cav-1 and interacting molecules regulates manifestation and maintenance of CSC properties remain to be fully understood. Because Cav-1 inhibits Nrf2 nuclear translocation, we speculated that the ability of Cav-1 to inhibit Nrf2 dependent signaling might promote stemness. As shown in **Fig. 3-5**, Cav-1 knockdown increased the expression of stemness signaling molecules, such as Bmi-1, Sox2, Oct3/4, and EMT markers, Snail and N-cadherin,

which was decreased by siRNA silencing of Nrf2. Thus, loss of Cav-1 promotes stemness in breast cancer by activating Nrf2.

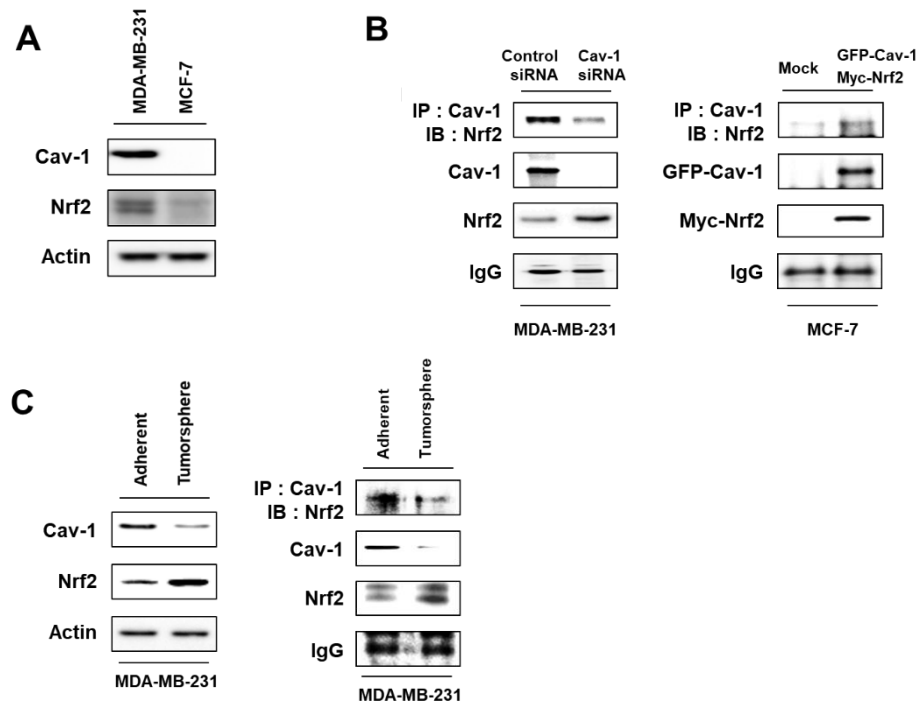

Fig. 3-1. Interaction between Cav-1 and Nrf2 in breast cancer cells. (A) The expression levels of Cav-1 and Nrf2 in breast cancer MDA-MB-231 and MCF-7 and cells were measured by Western blot analysis. **(B)** MDA-MB-231 cells were transfected with control siRNA or Cav-1 siRNA, and MCF-7 cells were transiently co-transfected with empty (MOCK) or GFP-tagged-Cav-1 or Myc-tagged-Nrf2 vector for 36 h. Immunoprecipitates with anti-Cav-1 antibody were immunoblotted for Nrf2. **(C)** MDA-MB-231 cells were cultured on ultra-low attachment plates for 5 days to generate primary tumorspheres, and then were dissociated into single cell suspension to generate secondary tumorspheres for 5 days. Using the same experimental method, tertiary tumorspheres were generated from secondary

tumorspheres. The expression of Cav-1 and Nrf2 was measured by Western blot analysis (left). Cav-1 immunoprecipitates were immunoblotted for Nrf2.

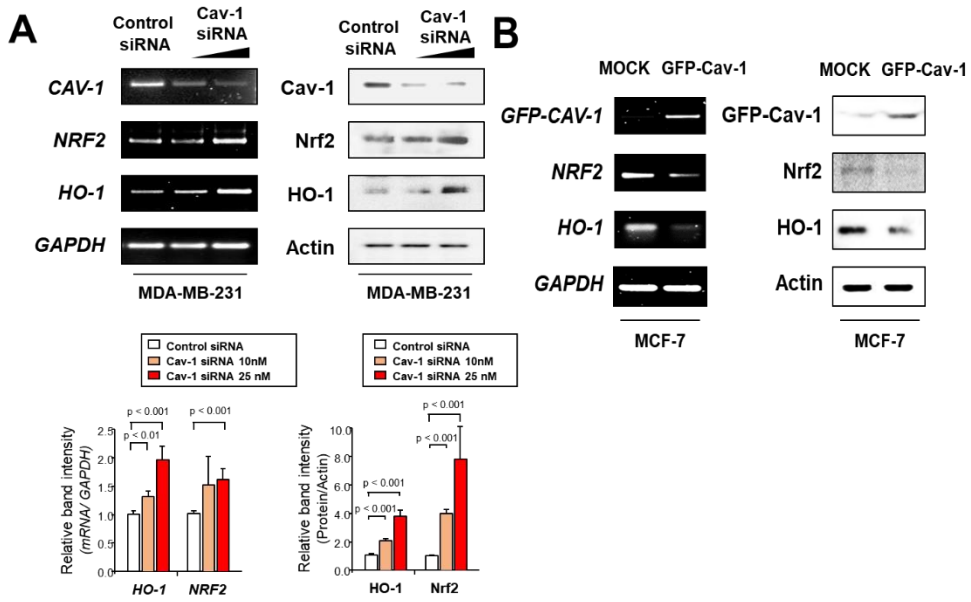

Fig. 3-2. Role of Cav-1 in regulation of Nrf2 activity. (A) MDA-MB-231 cells were transfected with control siRNA or Cav-1 siRNA for 36 h. The mRNA and protein levels of Nrf2 and HO-1 were determined by RT-PCR and Western blot analysis, respectively. The values are means ± SD (n = 3). **(B)** RT-PCR and Western blot analysis of Nrf2 and HO-1 in MCF-7 cells that were transfected with empty (MOCK) or GFP-tagged-Cav-1 vector for 36 h. GAPDH and β-actin were used as a loading control.

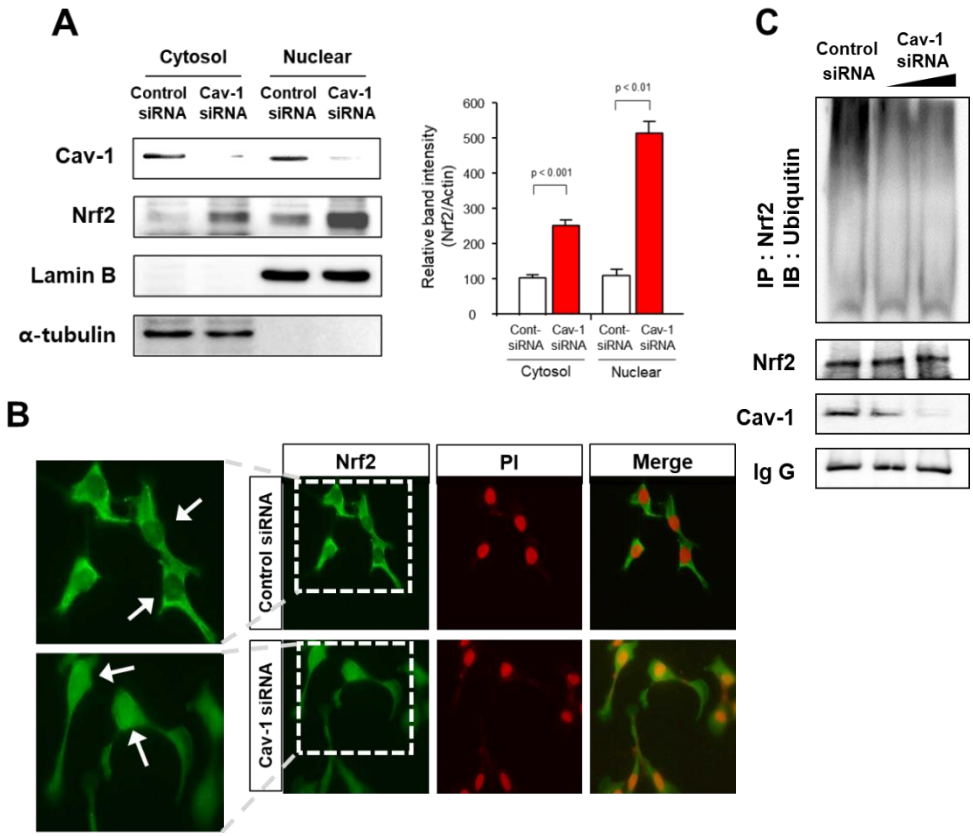

Fig. 3-3. Cav-1 and Nrf2 interaction in cytosol and nucleus. MDA-MB-231 cells were transfected with control siRNA or Cav-1 siRNA for 36 h. **(A)** The cytoplasmic and nuclear fractions were isolated as described in the Materials and methods. Both fractions were subjected for Western blot analysis of Cav-1 and Nrf2. The values are expressed as means ± SD (n = 3). **(B)** Immunocytochemical analysis was performed using antibody against Nrf2. Nuclei were identified by propidium iodide (PI) staining. **(C)** To detect the ubiquitination of Nrf2, cells were immunoprecipitated with anti-Cav-1 antibody, followed by Western blot analysis with anti-ubiquitin antibody.

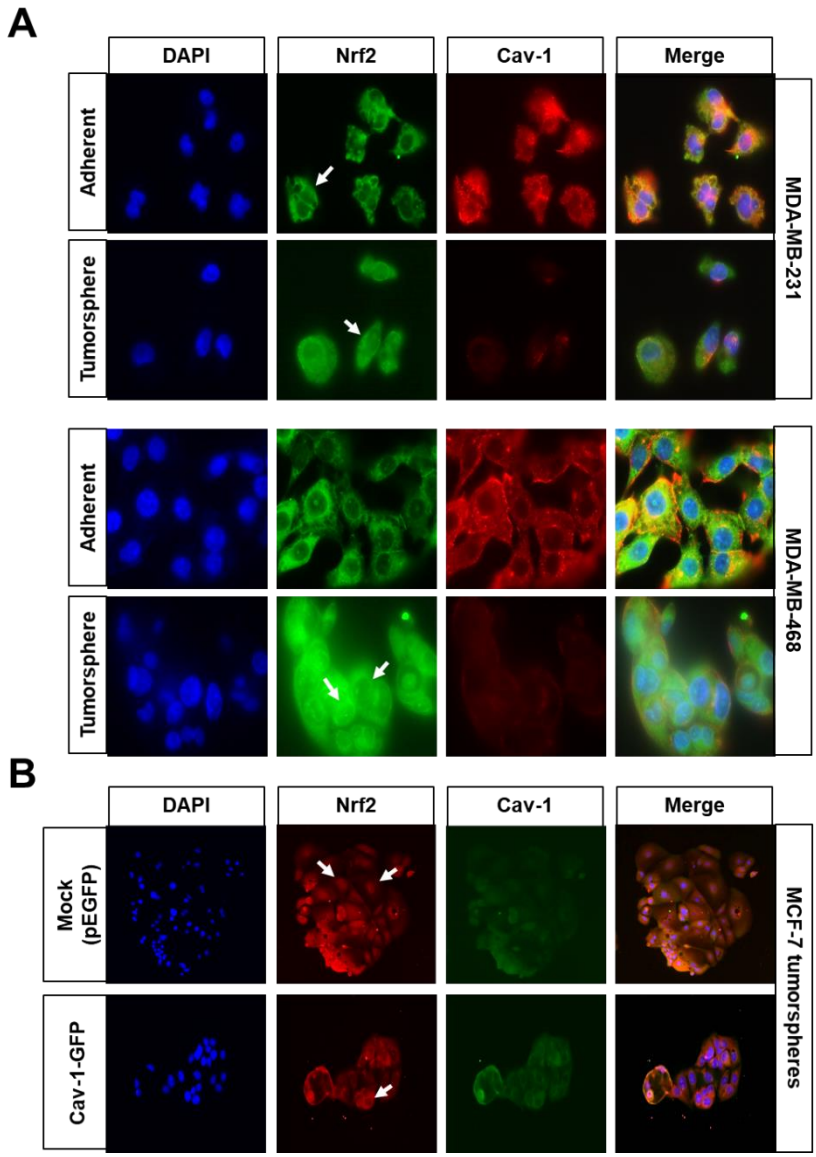

Fig. 3-4. Distribution of Cav-1 and Nrf2 in tumorspheres derived from human breast cancer cells. Immunocytochemical analysis was performed using antibodies against Cav-1 and Nrf2. Representative images were visualized under a microscope. **(A)** MDA-MB-231 and MDA-MB-468 cells were cultured on ultra-low attachment plates for 5 days to generate primary tumorspheres, and then were dissociated into

single cell suspension to generate secondary tumorspheres for 5 days. Using the same experimental method, tertiary tumorspheres were generated from secondary tumorspheres. **(B)** MCF-7 cells were transiently transfected with empty (MOCK) or GFP-tagged-Cav-1 vector for 36 h, and then tertiary MCF-7 tumorspheres were generated. Conditions for tertiary tumorspehres are same as described in the legend to (A).

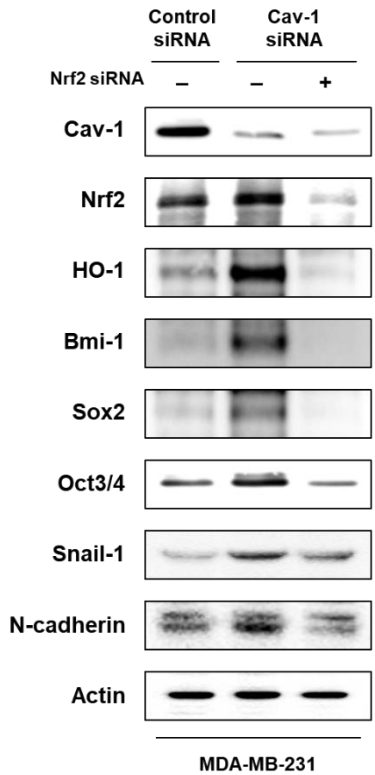

Fig. 3-5. Effects of Cav-1-mediated Nrf2 on manifestation of stem-like trait in MDA-MB-231 cells. MDA-MB-231 cells were transfected with control siRNA or Cav-1 siRNA, or co-transfected with Cav-1 siRNA and Nrf2 siRNA for 36 h. The protein levels of Cav-1, Nrf2, HO-1, Bmi-1, Sox2, Oct3/4, and EMT markers (Snail, N-cadherin) were determined by Western blot analysis.

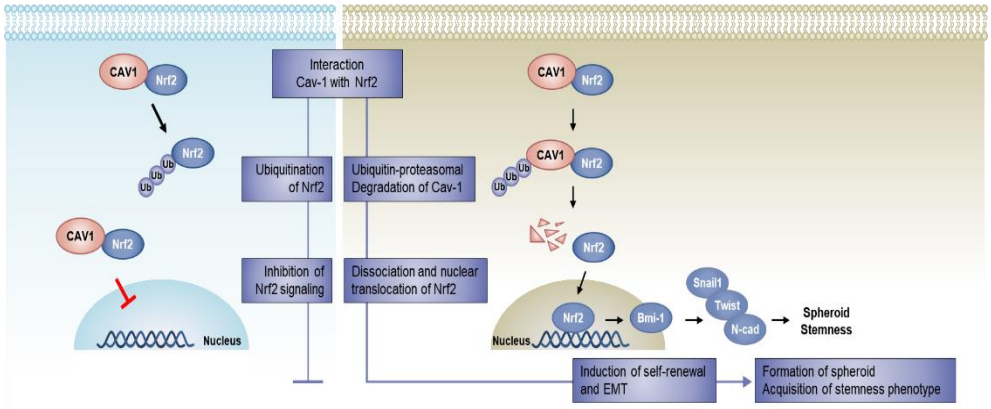

Fig. 3-6. Schematic representation of the role of Cav-1 and Nrf2 in regulating stem-like traits in breast cancer cells. Cav-1 functions as a endogenous inhibitor of Nrf2, thereby suppressing its activity and attenuating stem-like traits. Cav-1 binds to Nrf2 and enhances its degradation in cytosol through ubiquitination. The reduction of the intracellular accumulation of Nrf2 by Cav-1 leads to a decrease in stemness-related gene expression. When degradation of Cav-1 occurs through the ubiquitin-proteasomal degradation, Nrf2 is dissociated from the Cav-1 complex. The liberated Nrf2 accumulates in the nucleus and enhances the expression of stemness-related genes, which promote CSC-like phenotypes.

5. Discussion

In this study, I provided new evidence showing that Cav-1 inhibits manifestation of stem-like traits in breast cancer cells through direct binding with Nrf2. CSCs are defined as subset of cancer cells with self-renewal capacity. CSCs are responsible for tumor progression, metastasis and recurrence. Therefore, the regulation of CSCs is considered a fundamental approach to control the aggressiveness of tumors [27]. There are various intrinsic and extrinsic factors involved in controlling the stemness of cancer cells [28, 29]. The intrinsic factors include tumor suppressor proteins, oncoproteins, and epigenetic mutation, whereas the extrinsic factors include microenvironmental mediators, cytokines, and paracrines.

Cav-1 is an oncogenic membrane protein associated with cholesterol distribution, endocytosis, extracellular matrix organization, and various cellular signaling, especially in cancer metabolism [30, 31]. Therefore, Cav-1 has been proposed as potential therapeutic target for disrupting tumor progression and metastasis. Cav-1 acts as a scaffolding protein by interacting with and modulating the activity of caveolae-signaling molecules, such as H-Ras, Src family tyrosine kinases and eNOS [4, 32, 33]. In most cases, these caveolin interaction activities have an inhibitory effect, leading to inactivation of the signaling molecules and modulation of downstream signal transduction.

Nrf2 has been reported to interact with Cav-1 [17, 18], Nrf2 is a key transcription factor that regulates an adaptive stress response. Li and colleagues have reported the inhibitory effect of Cav-1 in Nrf2-mediated signaling [18]. Volonte *et al.* have demonstrated that Cav-1-mediated sequestration of Nrf2 promotes premature senescence by preventing excessive antioxidative signaling [17]. In the present study, I elucidated the function and underlying molecular mechanism of the Cav-1 and Nrf2 interaction which contributes to breast cancer stemness. In our previous study, the stability of Cav-1 was found to be controlled by Src-mediated phosphorylation, ubiquitination and degradation. After Cav-1 is degraded, Nrf2 is dissociated from Cav-1 complex, and the free Nrf2 accumulated in the nucleus.

Several studies reported that self-renewal is a critical for the maintenance of the CSC-like phenotype [34]. Among the stemness-associated factors, Bmi-1, Oct3/4 and Sox2 are of prime interest. Bmi-1 is the central protein involved in regulation of self-renewal signaling [21, 22], and Oct3/4 and Sox2 regulate self-renewal and tumorigenicity of stem-like cells [23, 24]. It has been reported that Bmi-1 is a self-renewal regulator in CSCs [35]. Kreso *et al.* [36] reported that the immunodeficient mice transplanted with Bmi-1 knockdown human colorectal cancer cells showed the reduced tumor growth as compared to those harboring the functional *Bmi* gene. Moreover, Sox-2 plays a crucial role in self-renewal activity mediated by epidermal

growth factor–receptor in pancreatic CSCs, and its overexpression enhances the proportion of breast CSCs by activating the Wnt signaling pathway [37, 38]. Furthermore, EMT is essential for generation of CSCs [25, 26]. Morel *et al.* showed that stem-like and tumorigenic properties of the cancer cells were driven by EMT [39]. In this context, self-renewal and EMT markers are considered the prominent factors in maintenance of the CSC-like phenotype. Further, Cav-1 knockdown exhibited the increased stemness signaling molecules, which was decreased by Nrf2 siRNA, indicating that Cav-1 regulates stem-like properties through Nrf2-mediated signaling.

Taken together, Cav-1 interacts with Nrf2 in breast cancer and inhibits Nrf2 nuclear translocation, thereby suppressing CSC-like properties. Therefore, Cav-1 is likely to function as a spontaneous inhibitor of Nrf2, purging stemness capacity of breast CSCs.

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Chapter IV

Effects of resveratrol on Caveolin-1-mediated manifestation of breast cancer stem-like properties

1. Abstract

While targeting cancer stem cells (CSCs) has arisen as an essential aspect, dietary phytochemicals with anticancer properties are promising candidates and have selective impact on CSCs. Resveratrol has been reported to exert chemopreventive and anti-carcinogenic effects on various cancers. In this study, I determined the effect of resveratrol on manifestation of breast cancer stem-like properties and explored the underlying molecular mechanisms, especially Cav-1-mediated signaling. Resveratrol inhibited cell viability in both MDA-MB-231 and MDA-MB-231 tumorspehres. Moreover, resveratrol reduced the tumorsphere forming ability and the proportion of $CD44^{high}$ and $CD24^{low}$ cells. Mechanically, the expression of self-renewal signaling molecules and EMT markers was reduced by treatment with resveratrol. Notably, resveratrol induced expression of Caveolin-1 in tumor spheres. Further, resveratrol-induced down-regulation of self-renewal and EMT markers was abrogated by knockdown of Cav-1. Taken together, these findings suggest that resveratrol inhibits the manifestation of breast cancer stem cell-like properties through suppression of Cav-1-mediated self-renewal signaling and EMT.

Keywords: Resveratrol; Caveolin-1; breast cancer stemness; self-renewal

2. Introduction

Cancer stem cells (CSCs), which is a small subset of tumor cells, are responsible for tumor-forming and self-renewing within cancer tissues [1]. Considering the tumor resistance to conventional therapeutic treatment, CSCs have attracted considerable attention for the development of new therapeutic strategies [2]. Among approved anticancer agents, almost one-third are either natural products or their derivatives [3], primarily from plants, seeds, and microorganisms [4].

Accumulating data have suggested that polyphenolic compounds, such as resveratrol, curcumin, and epigallocathechin gallate, exert anticarcinogenic effects by inhibiting tumor progression and metastasis and by inducing CSCs suppression [5-8]. Among the polyphenols, resveratrol (*trans*-3,4',5 trigydroxystilbene) has been extensively investigated with regards to biological properties including antioxidant, anti-inflammation, and antitumor capacity. Notably, resveratrol had the inhibitory effects on the invasion and metastasis of tumor cells through the EMT process [9, 10]. Furthermore, resveratrol inhibited self-renewal and invasive abilities of certain CSCs [11, 12]. Nonetheless, the underlying mechanism by which resveratrol regulates the signal transduction pathways involved in maintenance of breast CSCs and manifestation of their characteristics still remains to be largely explored.

Self-renewal is one of the essential features of the CSCs to maintain the proliferating capacities [13]. Bmi-1 and Notch-1 are known as representative regulators of self-renewal signaling [14, 15]. It is noticeable that CSCs are highly enriched with Bmi-1 and co expressed with Sox2, Oct3/4 and Nanog, which also play a role in the maintenance of CSCs [16, 17]. Several studies have demonstrated that induction of EMT in transformed epithelial cell was shown to culminate in endowing cells with acquiring stem-like traits, leading to tumor growth, invasiveness and metastasis [18, 19]. Based on these studies, self-renewal signaling molecules and EMT markers are widely used to identify the CSCs.

Caveolin-1 (Cav-1) is the major component of caveolae, which is flask-shaped invaginations present at the plasma membrane in a variety of cell types. Caveolae act as signaling platforms, serve as concentrating points for numerous signaling molecules, and regulate flux through many distinct signaling cascades [20]. Cav-1 regulates a variety of cellular events that include cellular transformation, tumorigenesis, angiogenesis, and cell metastasis [21-23]. Recently, it has been reported that Cav-1 is indispensable for regulation of CSCs [24]. In this study, I examined the effect of resveratrol on breast cancer stemness, especially in the context of its modulation of Cav-1 signaling.

3. Materials and methods

Reagent and antibodies

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). TRIzol® was obtained from Invitrogen (Carlsbad, CA, USA). Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for Bmi-1, Notch-1 and Sox-2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Cav-1, Oct 3/4, Snail, and β-actin were obtained from Santa Cruz (Santa Cruz, CA, USA). Antibodies against Twist1, N-cadherin, CD24 and CD44 were purchased from BD Biosciences (Bedford, MA, USA). The bicinchoninic acid (BCA) protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL, USA).

Cell culture

The human breast cancer cell lines MDA-MB-231 were obtained from the Korean Cell Line Bank (Seoul, South Korea). MDA-MB-231 cells were maintained in DMEM. Culture media were supplemented with 10% FBS and 1% antibiotics, and cells were grown at 37℃ with 5% CO_2 / 95 % air.

Tumorsphere culture

For tumorsphere formation from the adherent cells, single cells were cultured in a serum free DMEM/F12 medium supplemented with B27 (GIBCO), 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL basic fibroblast growth factor (b-FGF; PeproTech, Rocky Hill, NJ, USA) and 4 ng/mL heparin (Sigma-Aldrich). Primary tumorspheres were seeded at a density of 1 x 10^4 cells/mL in 100 mm ultralow attachment plates (Corning, NY, USA) for 5 consecutive days, and 2 mL of medium was added every third day. To culture secondary tumorspheres, primary tumorspheres were gently collected and dissociated into a single-cell suspension using 40 μm strainer. Single cells were counted and then seeded again for another 5 days with addition of 2 mL medium every third day. Using the same experimental method, tertiary mammospheres were generated from secondary mammospheres. The number of mammospheres formed (> 100 μm) was counted at indicated times under a microscope.

Measurement of cell viability

MDA-MB-231 cells and MDA-MB-231 tumorspheres were plated at a density of 1×10^5 cells/mL in 48-well plates and the cell viability was determined by the conventional MTT reduction assay. After 24 h incubation with resveratrol, cells were

treated with the MTT solution (final concentration 0.5 mg/mL) for 3 h at 37 ℃. The dark blue formazan crystals that formed in intact cells were solubilized by DMSO, and absorbance at 570 nm was measured with a microplate reader (Molecular devices, Sunnyvale, CA, USA). Results were expressed at the percentage of MTT reduction obtained in the treated cells, assuming that the absorbance of control cells was 100 %.

Cell migration assay

Cell migration was determined using the Culture-Inserts (2×0.22 cm^2; Ibidi, Regensburg, Germany). To create a wound gap, 100 μL of 1×10^5 cancer cells were seeded on the Culture-Inserts, which were gently removed using sterile tweezers following an overnight incubation. Cells were then exposed to resveratrol (50 μM) for 24 h. The progression of wound closure was monitored, and distance between gaps was measured under the microscope (Nikon,Tokyo, Japan). All assays were performed in triplicate.

Flow cytometry analysis

Cells were collected, washed with phosphate-buffered saline (PBS), and dissociated with Accutase solution (Sigma-Aldrich). Cells were then counted and washed with

PBS containing 2% FBS and 0.1% Tween-20. Cells were stained with CD24-PE and CD44-APC for 30 min at 4°C. After incubation, cells were collected and washed with PBS again. Cells were dissociated into single cells by using 40 μm strainer, and then the population of $CD44^{high}/CD24^{low}$ cells was measured using BD FACSCalibur (Becton Dickinson Biosciences, San Jose, USA).

Western blot analysis.

Whole cell lysate was prepared by scapping the cells in RIPA lysis buffer [150 mM NaCl, 0.5 % Triton x 100, 50 mM Tri-HCl (pH 7.4), 25 mM NaF, 20 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 0.1 mM phenylmethane sulfonyl fluoride (PMSF)] for 15 min on ice followed by centrifugation at 13,000 *g* for 15 min. The supernatant containing proteins was collected and stored at -70 ℃. For Western blot analysis, the protein concentration of whole cell lysates was measured by the using the BCA protein assay kit (Pierce, Rockford, IL, USA). Lysates from cells were separated by running through 8-12 % SDS-PAGE gel and transferred to the polyvinylidene difluoride (PVDF) membrane (Gelman Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5 % non-fat dry milk/TBST (Tris-buffered saline buffer containing 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated with the respective primary antibody diluted in TBST overnight 4°C. Blots

were rinsed three times with TBST at 10-min intervals followed by incubation with respective horseradish peroxidase conjugated secondary antibodies (rabbit, mouse or goat) in TBST for 1 h at room temperature. The blots were washed again three times with TBST. The band intensities in Western blotting were visualized with an enhanced chemiluminescent (ECL)detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and quantified with the LAS-4000 Image Analyzer (Fujifilm, Tokyo, Japan).

Transient transfection of siRNA

MDA-MB-231 tumorspheres were seeded at a density of 1 x 10^5 cells/mL in 100 mm dish in complete growth media. Cav-1 siRNA (25 nM) was transfected into MDA-MB-231 tumorspheres with lipofectamine RNAiMAX (Invitrogen) reagent according to the manufacturer's instructions. The target sequence for human Cav-1 siRNA was 5' -AGA CGA GCU GAG CGA GAA GCA UU3' (forward) and 5' -UGC UUC UCG CUC AGC UCG UCU UU-3' (reverse). siRNA oligonucleotide targeting for Cav-1 was purchased from Genolution Pharmaceuticals (Seoul, South Korea).

Statistical analysis

Data were represented as means of ± standard deviation (SD) at least three independent experiments. Statistical significance was determined by Student's *t*-test and a *p*-value of less than 0.05 was considered to be statistically significant.

4. Results

Resveratrol reduced the cell viability and migration of human breast cancer cells

Among the various methods that enable to enrich for stemness of cancer cells lines, tumorspheres culture has been predominantly used. I generated tumorsphers from human breast cancer MDA-MB-231 cells [25, 26]. To investigate the biological function of resveratrol, I examined the effects of resveratrol on the viability and migration capacity. As illustrated in **Fig. 4-1A**, resveratrol significantly decreased the viability of adherent and tumorsphere MDA-MB-231 cells. Moreover, a wound-healing assay showed the inhibitory effects of resveratrol on migration of breast cancer MDA-MB-231 cells (**Fig. 4-1B**).

Resveratrol attenuated stemness of breast cancer cells

I further explored the effects of resveratrol on manifestation of stemness properties of breast cancer cells. The mammosphere formation assay is widely used to identify stem cells based on self-renewal activity [27, 28]. As shown in **Fig. 4-2A**, resveratrol significantly decreased the number and the size of MDA-MB-231 tumorspheres in a concentration-dependent manner. These results indicate that resveratrol could inhibit self-renewal ability of stem-like breast cancer cells. Moreover, the different cell populations within a cancer can be identified according

to the signature of proteins expressed on the surface of a particular cell. For example, $CD44^{high}/CD24^{low}$ breast stem-like cancer cells possess capacity to promote tumorigenesis in breast cancer models [29]. the proportion of $CD44^{high}/CD24^{low}$ cell population was decreased by resveratrol treatment (**Fig. 4-2B**).

Resveratrol reduces the expression of self-renewal signaling molecules and EMT markers in breast cancer cells

Self-renewal is the predominant property of CSCs that accounts for the regeneration of tumor [30]. It has been reported that Bmi-1 and Notch-1 are representative regulators of self-renewal signaling [31, 32]. Sox-2 and Oct3/4 are also essential for maintaining self-renewal of tumor-initiating cells [33]. As shown in **Fig. 4-3A**, resveratrol treatment resulted in the alleviated expression of Bmi-1 and Notch-1 with concomitant downregulation of Sox2 and Oct 3/4. According to the studies on the link among EMT, CSC, and the metastasis of cancer cells, induction of EMT in transformed epithelial cells was shown to culminate in endowing cells with stem-like traits [18, 19]. Resveratrol attenuated the expression of Snail, Twist1 and N-cadherin, well-known EMT markers (**Fig. 4-3B**). These findings suggest that resveratrol is likely to modulate stem-like traits of breast cancer cells through inhibition of self-renewal signaling and EMT.

Resveratrol inhibits Cav-1-mediated stemness in MDA-MB-231 tumorspheres

Recently, Cav-1 and related signaling molecules have been suggested as a critical regulator of CSCs [24, 34]. In our previous studies, loss of Cav-1 promotes stemness properties in breast cancer cells through self-renewal signaling and EMT phenotype. Therefore, to verify the molecular mechanism for the inhibition of stemness properties of resveratrol, its effects on the expression of Cav-1 in MDA-MB-231 tumorspheres were examined. As shown in **Fig. 4-4A**, treatment with resveratrol restored the expression of Cav-1 which was constitutively down-regulated in MDA-MB-231 tumorspheres. However, the expression of Cav-1 in adherent cells was not altered by resveratrol treatment under the same experimental conditions. To validate whether the inhibitory effect of resveratrol on breast cancer stemness is mediated by upregulating Cav-1, MDA-MD-231 tumorspheres were transfected with transiently Cav-1 siRNA. Consistent with the above findings, MDA-MB-231 tumorspheres transfected with non-specific siRNA exhibited the increased protein expression of Bmi-1, Sox2, Oct3/4, and EMT markers upon resveratrol treatment. However, such effect of resveratrol on the expression of the aforementioned proteins was attenuated by Cav-1 siRNA transfection (**Fig. 4-4B**). These results support the notion that resveratrol impedes Cav-1-mediated stemness signaling in breast tumorspheres.

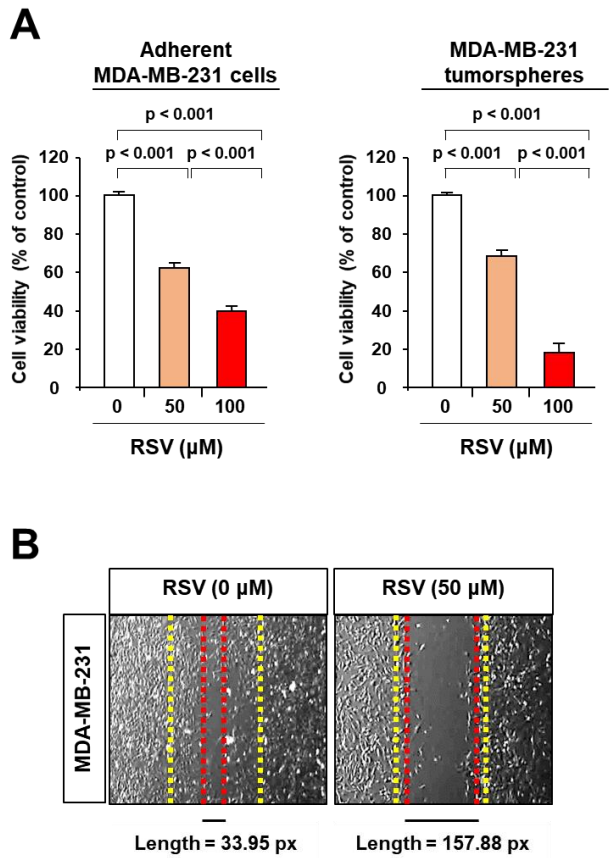

Fig. 4-1. Effects of resveratrol on viability and migration of breast cancer cells.

(A) The cell viability was examined by the MTT assay. Cells were treated with resveratrol (50 or 100 μM) or vehicle for 48 h. The cells were then incubated with MTT solution (0.5 mg/mL) for 2 h, and the absorbance at 570 nm was read using a microplate reader. The results are shown as the mean ± S.D. of triplicates. For tumorsphere culture, cells were cultured in an ultra-low attachment plate to generate primary tumorspheres. After 5 days, primary tumorspheres were counted, collected and replated under the same conditions to form secondary tumorspheres. Using the

same experimental method, tertiary mammospheres were generated from secondary mammospheres. **(B)** For the wound healing assay, MDA-MB-231 cells were treated with resveratrol for 48 h and were then plated into culture-insert (Ibidi). After 48 h incubation, cell migration was assessed by measuring the wound size under a microscope.

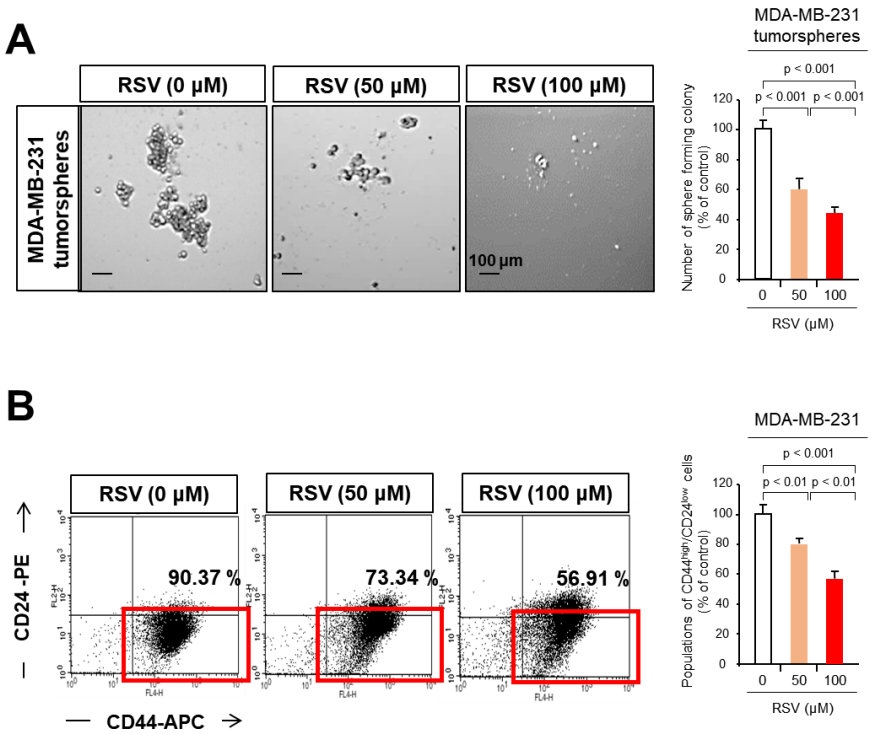

Fig. 4-2. Inhibitory effects of resveratrol on tumorsphere formation and manifestation of breast CSC properties. (A) Cells were cultured on ultra-low attachment plates for 5 days to generate primary tumorspheres, and then were dissociated into single cell suspension to generate secondary tumorspheres for 5 days. At the tertiary tumorspheres states, cells were cultured in a 96-well ultra-low attachment surface plate, treated with resveratrol (25 or 50 μM) for 5 days. Alterations in the number, the size and the shape of mammospheres were examined by phase-contrast microscopy. Representative images were visualized under a microscope. Scale bar = 100 μm. The values are presented as means ± SD. **(B)** Proportions of $CD44^{high}$ and $CD24^{low}$ cells were determined by flow cytometry in

the MDA-MB 231 cells treated with resveratrol (25 or 50 μM) for 48 h. The cells were stained with anti-CD44-APC and anti-CD24-PE antibodies. The values are expressed as means ± SD (n = 3).

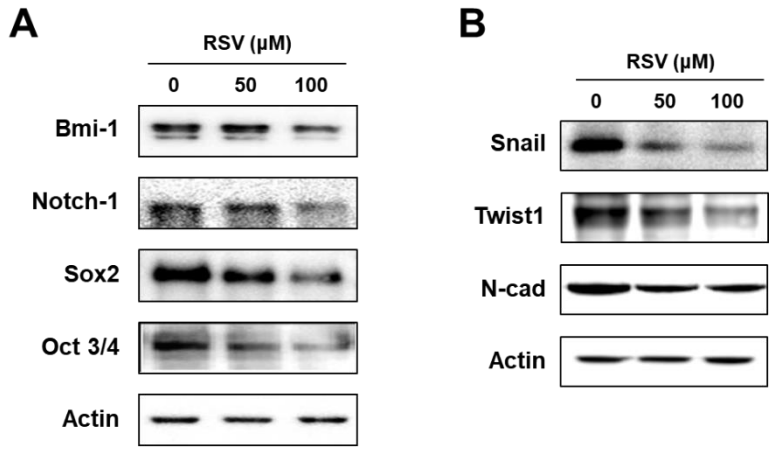

Fig. 4-3. Effects of resveratrol on expression of self-renewal signaling and EMT markers in breast cancer cells. MDA-MB-231 cells were cultured with or without resveratrol at indicated concentrations for 48 h. Western blot analysis was performed to measure the expression of **(A)** self-renewal markers, Bmi-1, Notch-1, Sox2, and Oct 3/4 and also **(B)** EMT markers, Snail, Twist and N-cadherin. β-actin was used as a loading control.

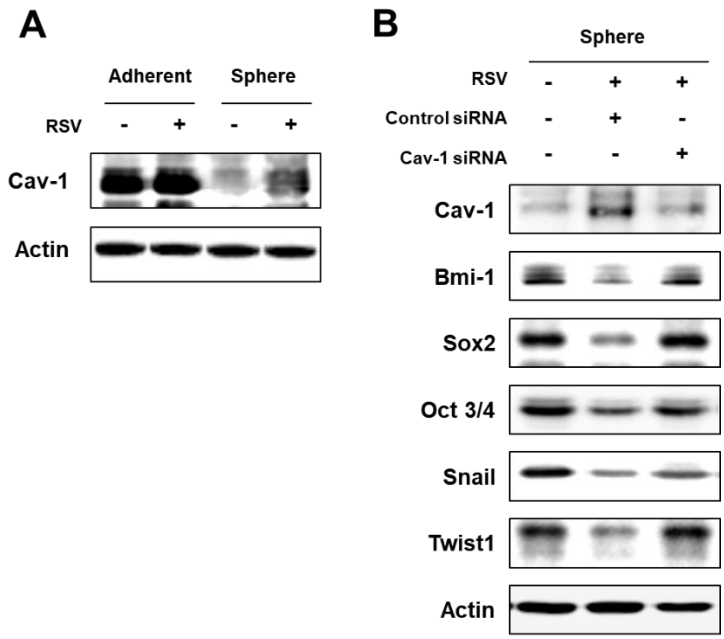

Fig. 4-4. Role of Cav-1 in inhibition by resveratrol of expression of signaling molecules involved in breast stem-like traits. MDA-MB-231 cells were cultured on ultra-low attachment plates for 5 days to generate primary tumorspheres, and then were dissociated into single cell suspension to generate secondary tumorspheres for 5 days. Using the same experimental method, tertiary tumorspheres were generated from secondary tumorspheres. **(A)** The expression level of Cav-1 was measured by Western blotting in adherent and tumorspheres treated with or without resveratrol (50 μM) **(B)** MDA-MB-231 tumorsphres transfected with non-specific or Cav-1 siRNA, followed by resveratrol treatment. Western blot analysis was performed to measure the expression of self-renewal signaling and EMT marker proteins as well as Cav-1. β-actin was used as a loading control.

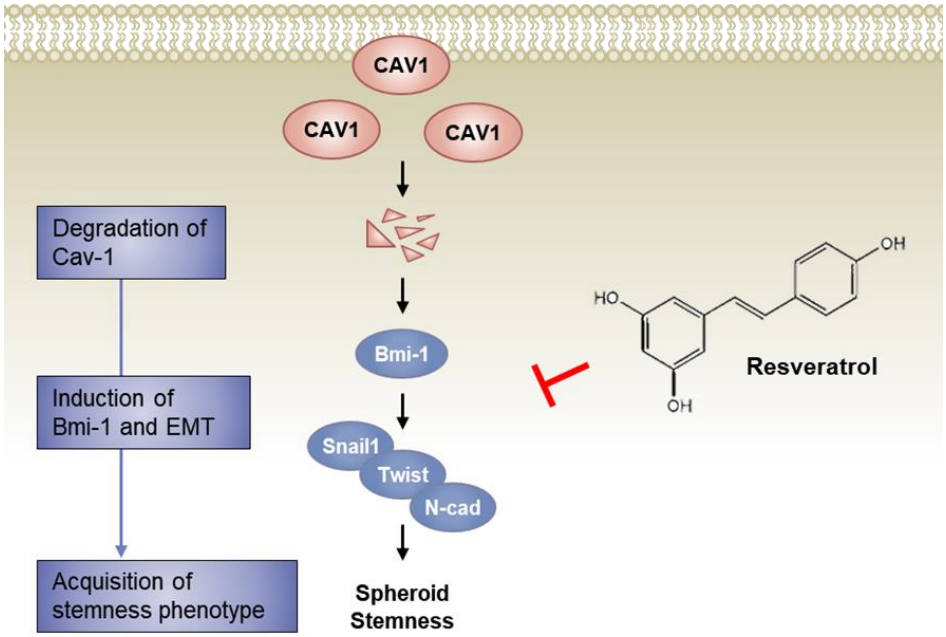

Fig. 4-5. Schematic representation of a proposed mechanism underlying the inhibitory effects of resveratrol on stem-like traits of breast cancer modulated by Cav-1.

5. Discussion

Cancer stem cells (CSCs) confer resistance to conventional chemotherapy, which often leads to recurrence in many different types of malignancies [2]. Therefore, targeting CSCs has been considered as one of the promising strategies for the development of next-generation anticancer drugs. Accumulating data suggest that phytochemicals, chemical compounds produced from natural sources, have the ability to damage CSCs and prevent tumor invasion and metastasis [4, 35].

Resveratrol is a polyphenolic compound extracted from plants, including grapes, mulberries, and peanuts. Several studies have suggested that resveratrol has diverse pharmacological activity activities, such as antioxidant, anti-inflammatory, antibacterial and anticancer against breast, prostate, skin, and colon [36, 37]. Resveratrol represses the growth of colorectal cancer cells through inhibition of Wnt signaling [38]. Resveratrol also impedes stemness in pancreatic cancer by inhibiting pluripotency maintaining factors and EMT [39]. Although there are a number of studies demonstrating the prominent efficacy of resveratrol in suppressing proliferation or growth of cancer cells, its effects on oncogenic potential of CSCs remains largely unknown.

Self-renewal capability is crucial for the maintenance of the CSC-like phenotype [13]. Several signal transduction pathways and their constituents, such as

Wnt, Notch, Hedgehog, Bmi-1, PI3K/AKT and IL6, are the important factors involved in regulations of self-renewal signaling [13]. It has been reported that Notch has emerged as a key regulator involving stem cell maintenance, cell-fate specification, and differentiation [40]. It has been reported that knockdown of Notch-1 enhances chemosensitivity and inhibits growth of human breast cancer [41]. In addition, Bmi-1 has been shown to be a key regulator of the self-renewal of many normal and cancer stem cells. Bmi-1 is overexpressed in breast cancer cells, regulating EMT and metastasis of cancer cells [42]. Moreover, transcription factors, Oct4, Nanog, and Sox2 play critical roles in maintaining the pluripotence and self-renewal characteristics of CSCs. Knockdown of Sox2 attenuated self-renewal capacity, and chemoresistance through ABCG2 suppression in head and neck squamous cell carcinoma [43]. Furthermore, EMT is associated with metastasis formation as well as with generation and maintenance of CSCs [18, 44]. Morel *et al.*, showed that stem and tumorigenic characters of the cells were driven by EMT [45]. In this context, self-renewal and EMT markers are considered the prominent factors in maintenance of the CSC-like phenotype.

Several recent studies have indicated that caveolin-1, a membrane transporter protein, is involved in mediating cancer invasion, metastasis and stem cell signal

transduction [46-48]. Cav-1 regulates self-renewal and invasive abilities in various cancer stem cells such as lung [49], breast [34], and pancreas [50].

Cav-1 expression is repressed in tumor spheres, which may account for the dormancy of CSCs. In this study, I found that resveratrol impeded stem-like traits of breast cancer through upregulation of Cav-1. The reduced Cav-1 protein expression in breast CSC state appears to be due to its destabilization through ubiquitin-proteasome degradation [51]. It has been known that Src-mediated phosphorylation of Cav-1 at the tyrosine 14 residue facilitates the proteasomal degradation of Cav-1. Further studies will be necessary whether resveratrol-induced restoration of Cav-1 in tumorspheres in breast cancer is mediated by suppressing the Src expression/activity.

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CONCLUSION

A small subpopulation of cancer cells, termed cancer stem cells (CSCs), have been proposed as a mechanism underlying chemo-resistance, tumor recurrence and metastasis. Therefore, ultimate cancer therapy is to develop an efficient strategy to eradicate CSCs through regulating signaling molecules. Cav-1 is a key regulator of cell signaling. Cav-1, which mediate cancer-relevant signaling transduction, has been proposed as potential therapeutic targets for disrupting tumor progression and metastasis.

This study showed that loss of Cav-1 led to the activation of signaling cascades, with stem-like traits, such as increased sphere formations and tumorigenicity. Reduced Cav-1 levels were attributable to its destabilization through ubiquitin-proteasome degradation. Hence, Cav-1 destabilization by Src-mediated phosphorylation may play a pivotal role in manifestation and maintenance of stemness in breast cancer cells.

Cav-1 might act as scaffolding proteins by directly interacting with and modulating the activity of signaling molecules. Here, Cav-1 interacted with Nrf2, suppressed the activity of Nrf2 and promoted proteasomal degradation under normal condition. When degradation of Cav-1 occurs through the ubiquitin-proteasomal

degradation, Nrf2 dissociated from Cav-1 and translocated to the nucleus, induced stemness properties. Therefore, Cav-1 functions as a spontaneous inhibitor of Nrf2, thereby suppressing its activity and attenuating subsequent stem-like traits.

It is reported that the use of naturally occurring compounds, especially phytochemicals, has gained enormous attentions because of a wide range of safety profile, ability to target multiple pathways in CSCs and their signaling pathways. Resveratrol, belongs to the group of polyphenols present in pigmented vegetables and fruits, causes an inhibitory effect on stem-like traits. Resveratrol inhibits the manifestation of breast cancer stem cell-like properties through suppression of Cav-1-mediated self-renewal signaling and EMT.

Taken together, reduced Cav-1 depends on its destabilization by Src through ubiquitin-proteasome degradation. After degradation of Cav-1, Nrf2 is dissociated from the Cav-1 complex. The liberated Nrf2 accumulates in the nucleus, and enhances the expression of stemness-related genes, which promote CSC-like traits. Resveratrol has an inhibitory effects on Cav-1-mediated CSC-like properties, which accounts for targeting CSCs in terms of suppression of cell migration, growth and signaling. This study herein indicates that Cav-1 acts as a pivotal regulator in manifestation and maintenance of stemness in breast cancer cells.

국 문 초 록

암줄기세포 (Cancer stem cell)는 종양의 개시 (initiation), 전이 (metastasis), 그리고 재발 (recurrence)에 있어 중요한 역할을 한다. 최근 연구 동향을 살펴보면, 암의 진행에 있어서 암줄기세포의 복잡한 생리학적 기능을 이해하고, 이들의 발생 기전을 조절하는 치료법 개발이 강조되고 있다. 본 연구에서는 그 발생 기전을 조절하는 조절인자 (modulator)로서 Caveolin-1 (Cav-1)을 선정하였다. Cav-1은 세포막 단백질인 Caveolae 를 구성하는 주요 단백질로써, 세포내 콜레스테롤 항상성 유지 (cholesterol homeostasis), 분자 수송 (vesicle trafficking), 암의 진행 (tumor progression) 및 신호 전달 (signal transduction) 등 다양한 기능에 관여한다. 그러나 Cav-1 의 암줄기세포에 대한 분자기전에 대한 연구는 미흡한 실정이다. 이에 본 연구에서는 암줄기세포의 발생기전에 있어서 Cav-1 의 역할에 대해 알아 보고자 하였다. 먼저, 줄기세포 배양방법으로 잘 알려진 sphere-forming culture system 을 통해 배양된 유방암 줄기세포 (tumorsphere MDA-MB-231)에서 Cav-1 의 발현이 낮아지는 것을 확인하였다. 유방암세포 MDA-MB-231 에 small interfering RNA (siRNA) 기법을 통해 Cav-1 을 억제하였을 때, 줄기세포에서 많이

나타나는 특성인 자가재생 (self-renewal)과 epithelial-mesenchymal transition (EMT)의 표현형 (phenotype)이 증가하고, 더불어 구체형성능 (tumorsphere forming capacity)이 증가하는 것을 확인하였다. 구체형성 세포 (tumorsphere cell)에서 Cav-1 의 발현이 낮아지는 것은 유비퀴틴화에 의한 단백질 분해 조절 기전임을 확인하였다. Cav-1 의 활성에는 tyrosine 14 번 잔기가 주로 역할을 하고 있다는 문헌 정보를 토대로, 특정 부위 돌연변이 (site-directed mutagenesis)를 통해 tyrosine 14 번을 phenylalanine 으로 치환하였을 경우, 자가재생능과 EMT 표현형이 감소되는 것을 통해, 줄기세포능을 유지하는데 있어서 Cav-1 의 tyrosine 14 잔기가 매우 중요한 역할을 하는 것을 확인하였다. 또한 Cav-1 이 다른 분자와 상호간의 직접적인 결합을 통해 신호 전달에 기여한다는 보고를 바탕으로 그 후보물질로서 Nrf2 를 선정하여, 실제 이들이 유방암 세포에서 결합을 이루고 있음을 확인하였고, tumorsphere cell 에서 Cav-1 의 단백질 안정화가 감소되면서 Cav-1 과 결합되어 있던 Nrf2 가 핵 안으로 이동되어 줄기세포능을 증가시키는 것을 관찰하였다.

최근 암줄기세포를 타겟으로 하는 암의 치료 및 예방을 위해 비교적 독성이 약한 식물 유래 화합물 (phytochemicals)을 이용한 연구가 많이 이루어 지고 있다. Resveratrol 은 그 가운데 대표적인 물질 중

하나로, 포도, 건과류 및 장과류 (berries) 등에 다량 함유되어 있는 폴리페놀 성분 물질로 항산화 효과, 심혈관계질환 예방효과, 항암효과, 염증관련 질환 예방 효과를 가진 것으로 보고되고 있다. Resveratrol 을 처리하였을 때, 유방암세포의 구체형성능과 자가재생, EMT 표현형이 감소하고, Cav-1 의 발현은 증가하는 것을 확인하였다. 이때 증가한 Cav-1 을 siRNA 를 이용하여 억제시키면, 감소하였던 줄기세포능이 다시 증가함을 확인하였다. 이와 같은 결과를 바탕으로 resveratrol 이 Cav-1 을 매개로 한 경로 조절을 통해 암줄기세포적 성격을 억제하여, 향후 암줄기세포를 타겟으로 하는 새로운 치료법의 접근이 가능할 것으로 사료된다.

주요어 (Key words)

Caveolin-1; Nrf2; Cancer stem cells; Signal transduction;

Resveratrol

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