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# Identifying the role of ornithine decarboxylase1 (ODC1) in breast cancer

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# Identifying the role of ornithine decarboxylase (ODC1) in breast cancer

Directed by Professor Joon Jeong

The Master's Thesis  
submitted to the Department of Medicine  
the Graduate School of Yonsei University  
in partial fulfillment of the requirements for the degree of  
Master of Medical Science

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This certifies that the Master's Thesis of  
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## TABLE OF CONTENTS

ABSTRACT .....	1
I. INTRODUCTION .....	2
II. MATERIALS AND METHODS	
1. Cell culture .....	8
2. Western blot analysis .....	8
3. Cell viability assay .....	9
4. Colony formation assay .....	9
5. Wound healing assay .....	10
6. siRNA transfection .....	10
7. qPCR .....	11
8. Apoptosis analysis .....	12
9. Cell cycle analysis .....	12
III. RESULTS	
1. ODC1 is highly expressed in breast cancer. ....	14
2. DFMO treatment highly reduced breast cancer cell growth. ....	15
3. DFMO treatment decreased colony formation ability and cell migration. .....	18
4. Decreased ODC1 expression through siRNA transfection .....	20
5. Reduced ODC1 expression in breast cancer cell induces apoptosis. .....	21
6. Downregulation of ODC1 in breast cancer cell induces cell cycle arrest at s phase. ....	23

IV. DISCUSSION .....	25
V. CONCLUSION .....	28
REFERENCES .....	29
ABSTRACT (IN KOREAN) .....	35

## LIST OF FIGURES

Figure 1. The polyamine metabolic pathway. ....	7
Figure 2. ODC1 expression level in breast cancer cell lines. ...	14
Figure 3. DFMO inhibits breast cancer cell growth. ....	16
Figure 4. DFMO inhibits cell colony formation and cell migration. .....	18
Figure 5. Downregulation of ODC1 by siRNA .....	20
Figure 6. Silencing of ODC1 expression by siODC1 induces apoptosis. ....	21
Figure 7. Silencing of ODC1 expression by siODC1 induces cell cycle arrest. ....	23

## LIST OF TABLES

Table 1. siRNA sequence .....	13
Table 2. Primer sequence .....	13

## ABSTRACT

**Identifying the role of ornithine decarboxylase 1 in breast cancer**

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(Directed by Professor Joon Jeong)

Ornithine decarboxylase 1 (ODC1) is a key enzyme involved in the production of putrescine in polyamine biosynthesis. ODC1 affects nucleic acid metabolism, cell growth, cell differentiation, and cell cycle. Increased ODC1 is associated with carcinogenesis, specifically in the invasion and angiogenesis phases. While several publications have suggested that the reduction of ODC1 inhibits cancer cell proliferation and metastasis, the mechanisms of cell growth regulation, proliferation, and invasion in breast cancer cells remain unclear. In this study, we investigated ODC1 expression levels in breast cancer cells. The ODC1 expression level of SKBR3 was highest among ten breast cancer cell lines. We treated SKBR3 with difluoromethylornithine (DFMO), which binds to ODC1 instead of pyridoxal phosphate to inhibit the action of ODC1. Cells in the treated group showed lower cell viability, cell growth, cell migration, and colony forming ability, compared to the untreated group. Down-regulated expression of ODC1 induced apoptosis and resulted in an accumulation of cells in the S phase of the cell cycle. Knock-down of ODC1 in breast cancer cells promoted the expression of cleaved Poly (ADP-ribose) polymerase, caspase-3, and caspase-9 and decreased expression of Bcl-2 in the mitochondrial pathway. The results of this study suggest that the polyamine pathway, including ODC1, has an important function in the cancer cell cycle, and when the expression of ODC1 is inhibited, cell cycle circulation is blocked, resulting in cell death, which inhibits the proliferation and metastasis of cancer cells. This study establishes the potential of ODC1 as target for the treatment of breast cancer. Further studies are needed to prove that ODC1 is a promising target for the treatment of breast cancer.

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Key words : breast cancer , ODC1 , DFMO , cell cycle, cell proliferation

# Identifying the role of ornithine decarboxylase 1 in breast cancer

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## I. INTRODUCTION

Breast cancer is the most common malignancy, accounting 30% of cancer diagnoses in women globally. It has the second- highest death rate after lung cancer.<sup>1</sup> Studies on factors that affect breast cancer (obesity<sup>2</sup>, long-term use of oral contraceptives<sup>3</sup>, dietary pattern<sup>4</sup>, genetic variations<sup>5,6</sup>, etc.) are very active. First, pathological remodeling of white adipose tissue and increased levels of fat-specific cytokines (mainly leptin), as a result of obesity, are associated with several hallmarks of breast cancer. These include sustained proliferative signaling, cellular energetics, inflammation, angiogenesis, activating invasion, and metastasis. Preclinical and clinical studies provide evidence that obesity may worsen the incidence, severity, and mortality of breast cancer.<sup>2</sup>

Second, the association between the use of oral contraceptive and breast cancer is controversial and a significant issue in public health. According to some report, the different mechanisms of action of various oral contraceptives, result in different carcinogenic effects. The use of oral contraceptives has been demonstrated to increase the risk of breast cancer occurring before age 35, and increased relative risks (RR) has been associated with long-term (over 10 years) use.<sup>7</sup> Thus, the associated risk of breast cancer differs between different types of oral contraceptives.<sup>8</sup>

Third, for dietary pattern, vegetarian have a lower risk of developing breast cancer than people who eat a diet containing meat.<sup>9</sup> Vegetarians and vegans eat a lot of soybeans and soy products, which are primarily isoflavones. Isoflavones are phytoestrogens, interacting with alpha- and beta-estrogen receptors to regulate estrogen levels in the body, and chemically prevent breast cancer along with serum albumin levels.<sup>10</sup> In addition, vegetables such as broccoli, cauliflower, cabbage, and kale help prevent breast cancer by acting as a substrate for glutathione transferase M1 and T1 detoxification enzymes and inducers.<sup>11</sup> And also consuming alcoholic drinks

associated with an increased risk of breast cancer. This finding was also published in the World Cancer Research Fund report. It has been hypothesized that alcohol consumption can lead to higher estrogen levels due to increased metabolites, which can interfere with the detoxification of carcinogens.<sup>12</sup>

Breast cancer is the most common genetic disease caused by the accumulation of modified genes. Both germline and somatic modifications have important roles in the development and progression of cancer.<sup>13</sup> For example, women with mutations in BRCA1 and BRCA2 are at greater risk of developing ovarian cancer as well as breast cancer. A deficiency or mutant form of BRCA 1 activates IGF1R, which promotes mitosis and anti-apoptosis roles through phosphatidylinositol-3-kinase and mitogen activating proteins, thereby increasing tumorigenesis.<sup>14</sup> Another example, the mutation of p53 is one of the frequent genetic modifications in breast cancer. When prolyl isomerase (Pin1) is overexpressed, it promotes the mutation of p53, which inhibits the expression of the antimetastatic factor p63, thereby promoting aggressiveness in breast cancer cells.<sup>15</sup> In addition, ATM and PALB2 are genes associated with germline mutation.<sup>16</sup> Some somatic mutations can activate the HER2 signaling pathway. HER2 somatic mutations occur more frequently in HER2-negative breast cancer, and HER2-negative breast cancer patients with these variants have less survival rate than wild-type HER2 patients.<sup>17</sup> ER pathway is involved during the progression of breast cancer to the invasion of other organs.<sup>18</sup> Somatic mutation of genes involved in ER pathway is an index that can predict a poor prognosis in ER-positive breast cancer.<sup>19</sup>

Breast cancer has been identified as a heterogeneous disease. The classification of breast cancer subtypes is divided according to the expression of estrogen receptor (ER), progesterone receptor (PR) or the human epidermal growth factor receptor 2 (HER2). There are three subtypes: HR + HER2-, HER2 +, and TNBC. The first HR + HER2- is when ER or PR is positive but HER2 is negative. The second HER2 + indicates that ER or PR is positive or both ER and PR are negative when HER2 is positive. Finally, it is called TNBC when all three of ER, PR, and HER2 are negative.<sup>20</sup> The breast cancer subtype is an important prognostic factor in breast cancer.<sup>21</sup> TNBC is an independent prognostic factor for breast cancer-specific survival with TNBC have worse clinical outcomes compared to those with other subtypes.<sup>22</sup>

Polyamines that are derived from amino acids are organic cations and are found in all

living organisms. Polyamine metabolism includes putrescine, spermidine, spermine. Under normal conditions, polyamine acts in the synthesis of proteins and nucleic acids through intercellular connections, cell metabolism, growth, and survival, and cell differentiation and death.<sup>23</sup>

Arginase 1 (ARG1) generates ornithine from the urea cycle. Regulation of polyamine homeostasis occurs through biosynthesis, which in most organism occurs via two pathways: the arginine decarboxylase pathway and the ornithine decarboxylase pathway. In the arginine decarboxylase pathway, polyamine biosynthesis starts with the change of arginine into ornithine, then into putrescine. The ornithine decarboxylase pathway involves the direct conversion of ornithine into putrescine.

Ornithine decarboxylase 1(ODC1) is a rate-limiting enzyme in polyamine biosynthesis which produces putrescine. Decarboxylated S- adenosylmethionine (dcAdoMet), a second rate-limiting enzyme in polyamine biosynthesis, is the amino propyl donor for spermidine synthase (SPDSY) and spermine synthase (SPMSY) for the synthesis of spermidine and spermine (Figure 1). The levels of ODC1, dcAdoMet, and spermidine/spermine N1-acetyltransferase 1 (SSAT) show obvious changes in response to elements related to cell growth and to alternations in polyamine levels. Vast alterations in enzyme activity can occur very quickly and are usually the result of alterations in multiple enzyme protein. Regulation can occur at the transcription, translation, and protein degradation stages.<sup>24</sup> ODC1 catalyzes the decarboxylation and conversion of ornithine to putrescine.<sup>25</sup> ODC1 is a pyridoxal phosphate (PLP)-dependent amino acid decarboxylase and it is a homodimer with two active sites each made up of residues from both subunits.<sup>26</sup> ODC1 has an important function as an antioxidant in DNA structure stabilization and double-strand cleavage pathways. It is an essential enzyme for cell growth, enabling the production of necessary polyamines. ODC1 has a short half-life and is regulated by transcription and translation, and its activity is regulated according to the G1 and G2 / M phases. When embryos deficient in ODC1 are implanted into the uterus, they do not develop properly. This suggests that ODC1 is necessary for cell survival.<sup>27</sup>

Polyamines, including ODC1, are overexpressed in many cancers. ODC1 overexpression is the most significant alteration in the polyamine metabolic pathway associated with cancer progression. Increased ODC1 activity is related to the acquisition of a hormone-independent, poorly differentiated phenotype in some breast cancers.<sup>28</sup> ODC1 activity is

higher in basal cell carcinomas than in normal human skin<sup>29</sup> and is significantly increased in prostate cancer compared with paired benign tissue.<sup>30</sup> The risk of cancer recurrence and death is higher in breast cancer patients with higher ODC1 activity than in those with fibrous breast tissue.<sup>31</sup> In endometrial cancer, increased levels of ODC1 are observed in association with high grade, late-stage, serous histology which are associated with increased rates of recurrence and death.<sup>32</sup> Upregulated ODC1 expression levels in esophageal squamous cell carcinoma significantly coincides with lymph node metastasis status and clinical stage.<sup>33</sup> Increased polyamines, including ODC1, are associated with carcinogenesis and are essential for invasion and angiogenesis related to increased cancer cell proliferation and metastasis.<sup>34</sup> Increased expression of ODC1 in NIH3T3 cells induces cell transformation, anchorage-independent growth in soft agar.<sup>35</sup> ODC1 levels have a function in promoting squamous cell carcinomas by driving continued proliferation.<sup>36</sup>

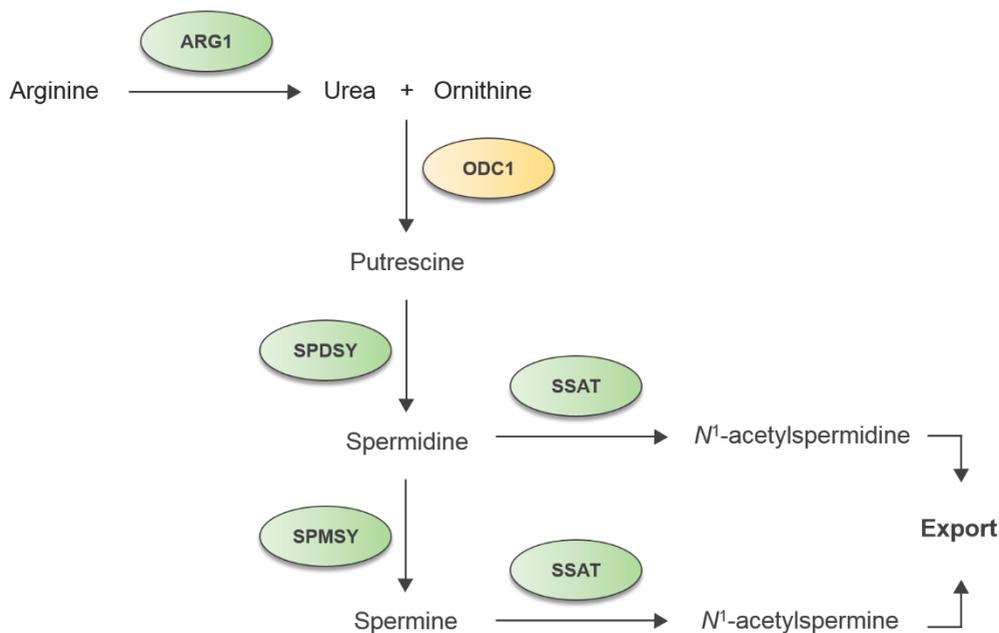
Studies are underway to treat polyamines, including ODC1, in several cancers. The relationship between polyamines, cell cycle arrest, and apoptosis has been studied in several cancers. In the G1 phase, during cell cycle, cyclin D1 binds to cyclin-dependent kinase 4 (CDK4) and regulates phosphorylation of retinoblastoma protein (pRB). pRB has an essential function in the transition from G1 to S phase. The transcription factor E2F transcription factor 1 (E2F-1) stimulates cyclin E transcription. Increased release of E2F-1 from pRB and stimulation cyclin A are involved in S phase progression. CDK1 binds to cyclin B to form a complex, which rapidly increases its activity, enters mitosis, and terminates cyclin B.<sup>37</sup> Reduction of ODC1 activity and polyamines results in cell cycle arrest in the S phase. Overexpression of SSAT in human prostate cells, leads to polyamine reduction, resulting in cell accumulation in the G2 / M stage.<sup>38</sup>

Cells in early apoptosis are associated with mitochondrial changes mediated by anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins. Bax activates the release of cytochrome c from the mitochondria, activating the caspase cascade.<sup>39</sup> When expression of ODC1 is decreased in esophageal cancer cells, apoptosis increases due to an increase in expression of cleaved caspase-3, cleaved PARP, and Bax.<sup>33</sup>

Increased polyamine levels are linked to skin cancer, prostate cancer, breast cancer, lung cancer, and crystalline cancer. Chemotherapy of polyamine pathways have been conducted by many researchers. Methylglyoxalbisguanylhydrazone and SAM486A are drugs which target decarboxylated S-adenosyl methionine (dcAdoMet), AMXT1501 and

TrimerPTI are drugs that inhibit polyamine transport.<sup>34</sup> Among the various polyamine metabolic inhibitors, difluoromethylornithine (DFMO) is an inhibitor of ODC1 which prevents ODC1 from being permanently fixed at the active site by strongly binding to instead of pyridoxal phosphate (an ODC1 cofactor). Without ODC1 activity, putrescine is depleted, causing problems in DNA and RNA synthesis, and inhibiting cell growth and division.<sup>23</sup> In several cancers, including neuronal carcinoma; skin cancer; endometrial cancer; liver cancer; and rectal cancer, the inhibition of ODC1 through DFMO is an effective means of treatment or prevention.<sup>40-42</sup>

ODC1 and polyamine biosynthesis has a critical function in the development of many cancers. Thus, some studies targeting this enzyme have been conducted. However, no studies have been conducted to determine the role of ODC1 in breast cancer. To investigate the role of ODC1 in breast cancer, here we use small interfering RNA(siRNA) and ODC1 inhibition to determine what phenotypes are shown when ODC1 expression is suppressed.



**Figure 1. Polyamine biosynthesis pathway.**

Arginase1 (ARG1) acts to make urea and ornithine from arginine. Ornithine decarboxylase 1 has a role in making putrescine from ornithine. Spermidine and spermine are produced by spermidine synthase (SPDSY) and spermine synthase (SPMSY), respectively. Spermidine/spermine N1-acetyltransferase 1 (SSAT) regulates the cellular content of polyamines by catalyzing acetylation of spermidine and spermine and exporting it out.<sup>23</sup>

## II. MATERIALS AND METHODS

### 1. Cell culture

All cells used in the experiment were maintained in RPMI 1640 medium (Thermo Scientific, Waltham, MA USA) supplemented with 10% fetal bovine serum(FBS, Biowest, Kansas city, Missouri USA) and 1% antibiotic/antimycotic (GIBCO, Thermo Scientific, Waltham, MA USA). Cells were cultured at 37°C in 5% CO<sub>2</sub> incubator. For siRNA transfection, cells were maintained in RPMI1640 supplement with 10% FBS, without antibiotics/ antimycotic for high efficiency

### 2. Western blot analysis

The western blot is a widely used analytical method in molecular biology, immunogenetics, and other disciplines to detect specific proteins in a sample of tissue homogenate or extract. The proteins are denatured, followed by gel electrophoresis. Secondary antibodies are added and the primary antibody recognizes and binds to their target protein. Secondary antibodies can be visualized through staining or fluorescence to detect the desired target protein.

Whole cells were lysed in 1x Radioimmunoprecipitation assay (RIPA) lysis buffer (Rockland, Pottstown, PA USA) on ice for 15minutes. Lysates were collected and supernatant was obtained by centrifugation at 12,000 rpm, 4°C for 15minutes. To determine protein concentration, Pierce™ BCA Protein Assay (Thermo Scientific, Waltham, MA USA) was performed. Then protein was mixed with 4x protein sample buffer(BIORAD, Contra Costa County, CA USA) and 2-mercaptoethanol was boiled at 100°C for 5minutes. 2-mercaptoethanol acts on the sample and breaks the SS bond and electrophoresis by converting the protein of dimer or more into monomer state. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was transferred to the polyvinylidene fluoride (PVDF) membrane. Unlike nitrocellulose and nylon membranes, PVDF is a hydrophobic material and does not adhere to a protein unless activated with methanol. Transferred membranes were treated with 5% skim milk in tris buffered saline with tween 20 (TBST) for 2hours at room temperature. Primary antibodies were reacted overnight at 4°C. The primary antibodies against ornithine decarboxylase (Catalog No. ab-126590), β-actin (Catalog No. ab- 8227) were purchased from abcam.

The primary antibodies to detect caspase-3 (Catalog No. 9662s), and caspase-9 (Catalog No. 9502s) were obtained from Cell Signaling Biotechnology and Bax (Catalog No. sc-7480), Bcl-2 (Catalog No. sc-7382), and cyclin A (Catalog No. sc-271682) antibodies were from santa cruz biotechnology. After washing five times with TBST, secondary antibodies were treated for 2hrs at room temperature. After chemoluminescence detection using the Electrochemiluminescence (ECL) solution (BIORAD, Contra Costa County, CA USA), blots were developed by image count LAS 4000.

### 3. Cell viability assay

Cells were plated in a 96-well plate (SPL LIFE SCIENCE) at a density of  $1 \times 10^4$  cells/well in triplicate and after 24hours, treated with different concentrations of (0mM to 5mM) difluoromethylornithine (Sigma, St.Louis, Missouri USA) in culture media. After incubation for 24, 48, and 72 hours, the supernatant was suctioned and the cells were treated with Cell Counting Kit-8 assay (Dojindo laboratories, Kumamoto, Japan), and incubated for 2 hours. Cell viability was determined at OD<sub>450nm</sub> with a plate reader (VersaMax, Molecular Device, Sunnyvale, CA, USA). A Cell Counting Kit-8 (CCK-8) was used for cell proliferation and cytotoxicity assays. Water-soluble tetrazolium salt (WST-8), a major component of CCK-8, was reduced by dehydrogenase in living cells to produce an orange form (formazan). The amount of formazan dye produced was directly proportional to the number of living cells.

### 4. Colony formation assay

The colony formation assay is an in vitro cell survival assay which measures the colony forming ability of a single cell to grow into a colony. Colony formation is the ability to cell division indefinitely. Leaking colonies comprise of at least 50 cells. Unlike normal cells, cancer cells do not die even if they grow excessively and grow in multiple layers beyond the monolayer membrane. In soft agar, cancer cells grow well without attaching to the ground due to anchorage-independent growth characteristics. The colony formation assay can be used to determine the effectiveness of cytotoxic agents.<sup>43</sup> Cells were plated in a 6-well plate (SPL LIFE SCIENCE) at a density of  $1 \times 10^4$  cells/well and after 24hours, treated with 0, 1.25, 2.5, and 5mM of DFMO in culture media. After one week, we changed the media containing DFMO and the cells were incubated for a total of 2 weeks

at 37°C. After 2 weeks, colony formation ability was photographed with a microscope mounted camera (ZIESS, Oberkochen, Germany), for observing the colony formation ability.

#### 5. Wound healing assay

The wound healing assay is an *in vitro* experiment used to examine collective cell migration in two dimensions. Exposure to a cell-free area induces cells to migrate into the gap. Migration is showed by epithelial and endothelial monolayers that move in two dimensions while maintaining their intercellular junctions and migration occurs in diverse processes such as cancer metastasis, embryonic morphogenesis, and tissue injury.<sup>44</sup>

In this experiment, the monolayer was scratched with a pipette tip and migration into the gap was imaged over several hours using a microscope equipped for live-cell imaging. Cells were plated in a 6-well plate at a density of  $1 \times 10^5$  cells/well and after 24 hours, treated with 0, 1.25, 2.5, and 5 mM of DFMO. After 4 days, DFMO-treated cells were harvested and seeded into culture-insert 2-well dish (ibidi, Nürnberg, Germany). When cell density reached 90~100%, the culture-insert 2-well was removed and the dish was filled with culture media containing either 0, 1.25, 2.5, and 5 mM of DFMO. Wound recovery changes were photographed with a microscope mounted camera (ZIESS, Oberkochen, Germany).

#### 6. siRNA transfection

siRNA binds to RNA-induced silencing complex (RISC), resulting in strands with a stable 5'-end. The single-stranded siRNA component then guides and combines the RISC on the target mRNA through the action of catalytic RISC protein.<sup>45</sup>

Lipofectamine is used to increase the transfection efficiency of RNA (including mRNA and siRNA) or plasmid DNA into the *in vitro* cell. Lipofectamine has a positively charged liposome formulation and forms a complex with a negatively charged nucleic acid molecule to penetrate the cell membrane. For expression of the transgene of a cell, the nucleic acid must extend to the nucleus of the cell to begin transcription. However, transfected genetic material is interrupted during the delivery process and cannot reach the nucleus from the transcription start. During cell division, the material may reach the nucleus by being trapped in the reassembling nuclear envelope following mitosis.<sup>46</sup>

siRNAs targeting ODC1 and non-targeting siRNA(scramble) were purchased from

BIONEER. We seeded SKBR3 cells by  $5 \times 10^5$  cells per 6-well plate and proceed with reverse transfection. To improve transfection efficiency, Lipofectamine 2000 (Invitrogen, Thermo Scientific, Waltham, MA USA) was diluted in Opti-mem reduced serum medium (GIBCO, Thermo Scientific, Waltham, MA USA) and 200 pmol siRNA was also diluted in Opti-mem medium. Mix 1: 1 with lipofectamine 2000 and siRNA diluted in Opti-mem reduced serum medium. The mixture was then incubated for 20min at room temperature. siRNA with Lipofectamine 2000 was transfected onto SKBR3. ODC1 expression was measured by RT-PCR and western blot. Targeted siRNA sequence information is shown in Table 1.

### 7.qPCR

Total RNA was extracted from the cells with TRIzol™ Reagent (Thermo Scientific, Waltham, MA USA) according to the manufacturer's instructions. In brief, growth media was removed and TRIzol™ Reagent was added directly to the culture dish by pipetting, to lyse the cells and homogenize the lysate. The lysate was centrifuged and the clear supernatant was transferred to a new tube. Chloroform was added to the mixture in order to separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase. Isopropanol was added, so that total RNA precipitate formed a white gel-like pellet at the bottom of the tube. Finally, the pellet was resuspended in 75% ethanol for washing. cDNA was produced using a cDNA Synthesis Kit (Thermo scientific, Waltham, MA USA). It is capable of synthesizing cDNA up to 20 kb from a wide range of total RNA amounts (1 pg to 5 µg) at elevated temperatures (45-65 °C). The reaction product of the first strand cDNA synthesis can be used directly in qPCR.

The PCR process consists of a series of temperature changes that are repeated 25–50 times. These cycles normally consist of three stages. First, at a temperature of around 95 °C, the nucleic acids double chain is separated. Second, at a temperature of around 50–60 °C, the primers are bound with the DNA template. Third, at between 68–72 °C, DNA polymerase is carried out.<sup>47,48</sup> To process PCR, we need to DNA polymerase, dNTPs, template, and primer, and anything else. For qPCR, we use SYBR Green mix containing dNTPs, MgCl<sub>2</sub>, and DNA polymerase. SYBR Green is a reagent that displays fluorescence by binding to double-stranded DNA. It binds to double-stranded DNA synthesized by a PCR reaction to emit fluorescence, and we can detect the fluorescence

sensitivity to measure the amount of amplified products produced.

The qPCR conditions were 95 °C/5min for one cycle, 95 °C/15sec and 60 °C/ 30sec and 72 °C/ 45sec for 30cycles, followed by 95 °C/ 15sec, 60 °C/1min, 95 °C/ 15sec 60 °C/ 15sec for one cycle. ODC1 expression levels were represented relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used in the study are listed in Table 2.

#### 8. Apoptosis analysis.

To identify apoptotic cells, cells were detected by flow cytometry using the FITC annexin V apoptosis detection kit (BD Biosciences, Bergen County, NJ, USA) according to the manufacturer's instruments. In apoptotic cells, membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V is a Ca<sup>2+</sup> dependent phospholipid-binding protein with a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.

Thirty hours after siODC1 transfection, cells were harvested by scraper and washed with 1x phosphate-buffed saline (PBS) and resuspended in the 1x binding buffer. Annexin V and propidium iodide(PI) mix was added to the cells and mixed lightly. The mixture was incubated for 15minutes at room temperature in the dark, and apoptosis detection was performed using FACSDiva flow (BD Biosciences, Bergen County, NJ, USA).

#### 9. Cell cycle analysis

For determine of cell cycle arrest cells, cells were recognized by flow cytometry using the PI solution in FITC annexin V apoptosis detection kit (BD Biosciences, Bergen County, NJ, USA). The fluorescence intensity of cells stained with PI is related to the amount of DNA the cells contain. DNA content in each cell cycle is often expressed as a histogram, which provides information about the relative frequency of cells.

Thirty hours after siODC1 transfection, cells were harvested by scraper and washed with 1x phosphate-buffered saline (PBS) and resuspended in the 1x binding buffer. Cells were stained with PI (50ug/ml) for 15minutes at room temperature in the dark. Cell cycle analysis was performed using FACSDiva flow (BD Biosciences, Bergen County, NJ, USA).

**Table1. siODC1 sequence**

siODC1 number	sense	antisense
1	GAC UAG GAU AUG GGU CAC A	UGU GAC CCA UAU CCU AGU C
2	GAG AUC ACC GGC GUA AUC A	UGA UCA CGC CGG UCA UCU C
3	CGA CGA UCC ACC AUG UGA U	AUC ACA UAG UAG AUC GUC G
4	CUG CCA CUU CCU CGA UGA A	UCC AUC GAG GAA GUG GCA G
5	GAC UGU GCU AGC AAG ACU G	CAG UCU UGC UAG CAC AGU C

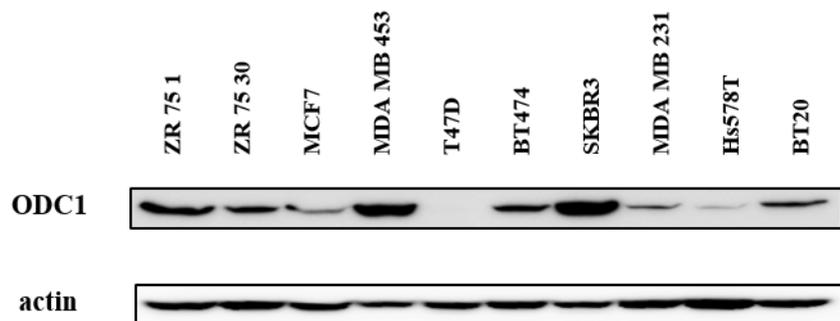
**Table2. Primer sequence**

Name	Primer sequence
ODC1	F : 5'-GAC GAG TTT GAC TGC CAC ATC-3'
	R : 5'-CGC AAC ATA GAA CGC ATC CTT-3'
GAPDH	F : 5'-GAG TCA ACG CAT TTG GTC GT-3'
	R : 5'-GAT CTC GCT CCT GGA AGA TG-3'

### III. RESULTS

#### 1. ODC1 expression level in breast cancer.

We used western blotting to qualify ODC1 protein in ten breast cancer cell lines. The results show that SKBR3 had the highest ODC1 expression level while MCF7, T47D, and Hs578T had lowest. (Figure 2) We selected SKBR3 for treatment with DFMO and ODC1 siRNA transfection. Due to their relatively high levels of ODC1 protein expression.



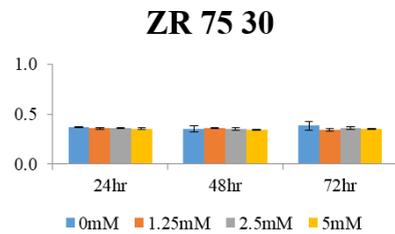
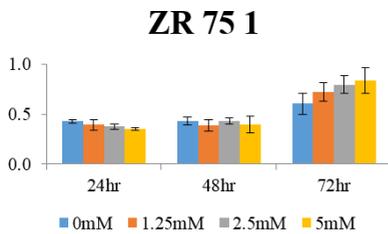
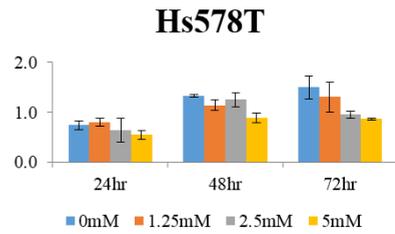
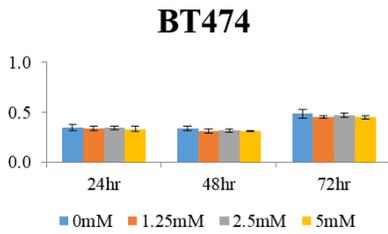
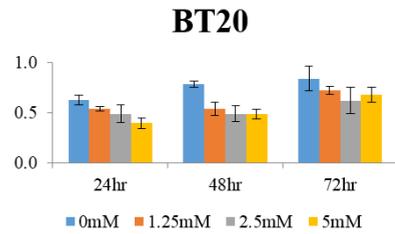
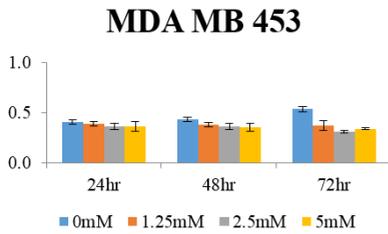
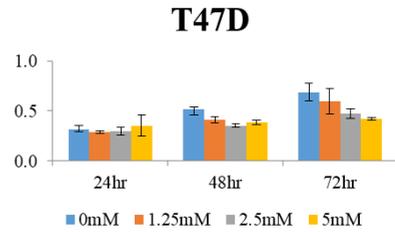
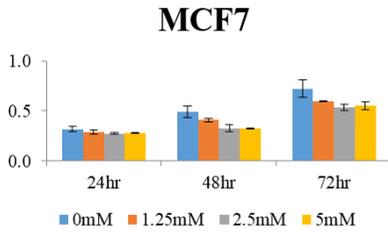
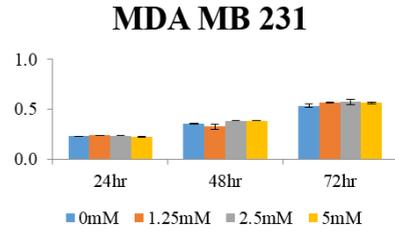
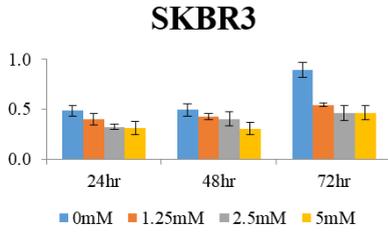
**Figure 2. ODC1 expression level in breast cancer**

Confluent monolayers of ZR-75-1, ZR-75-30, MCF-7, MDA-MB-453, T-47D, BT-474, SKBR3, MDA-MB-231, Hs578T, BT-20 were extracted with RIPA lysis buffer. 15 mg total protein from each cell extract was resolved by SDS-PAGE, transferred to PVDF, and blotted with antibodies against ODC1.

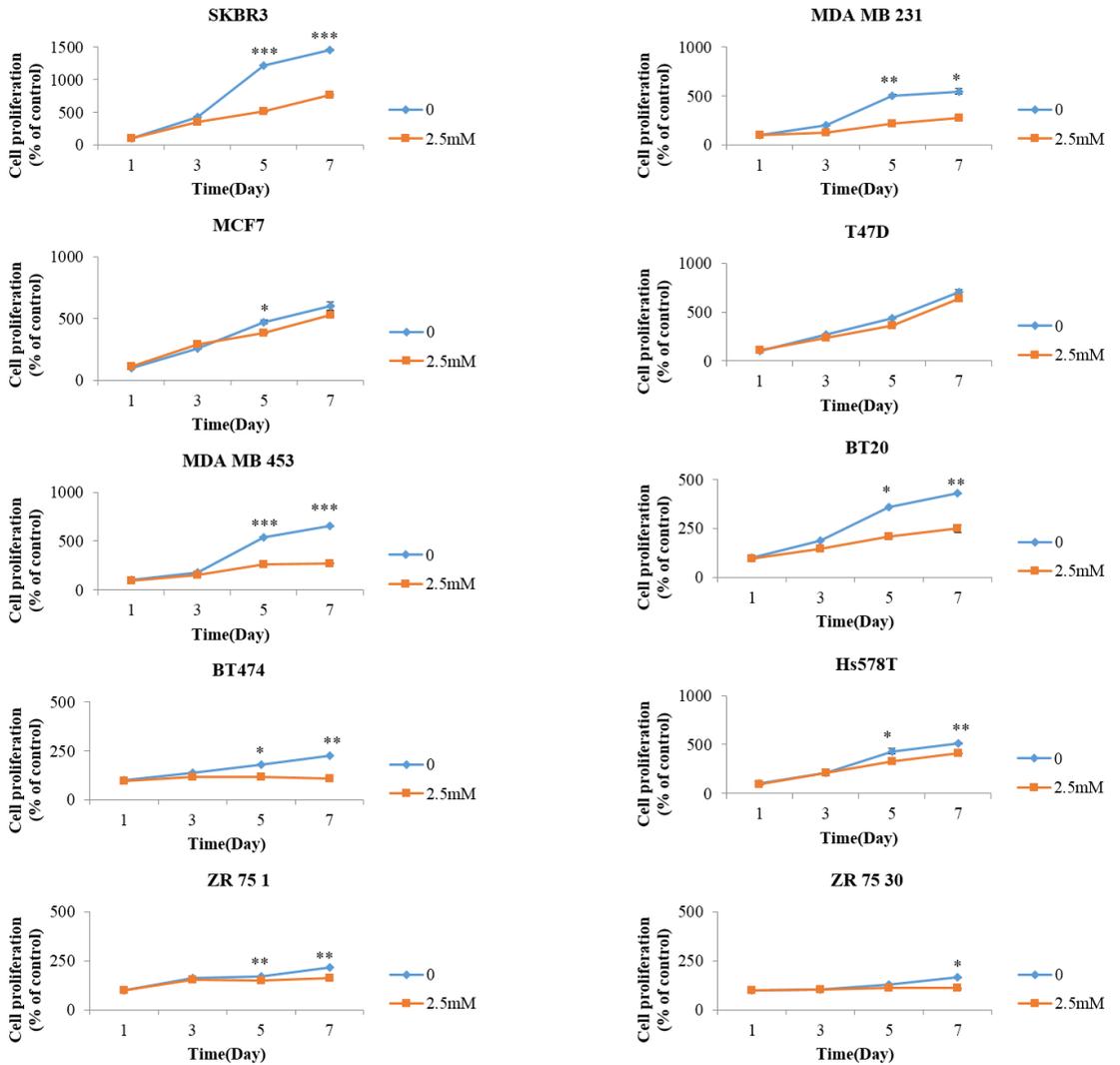
## 2. DFMO treatment highly reduced breast cancer cell growth.

We investigated whether DFMO inhibits cell growth by measuring the degree of ODC1 expression. SKBR3 and MDA MB 453, which had high ODC1 expression, was sensitive to low DFMO (1.25mM). MDA MB 231, T47D, and MCF7, which had low ODC1 expression, were less sensitive to high DFMO concentration. As an exception, BT474 and ZR 75 30 did not respond to the high concentration of DFMO (Figure 3a). Also, the growth of SKBR3 and MDA MB 453 treated with 2.5mM DFMO for 7 days decreased significantly after 3 days. Whereas the other cells showed slight differences or no difference at all (Figure 3b).

(a)



(b)



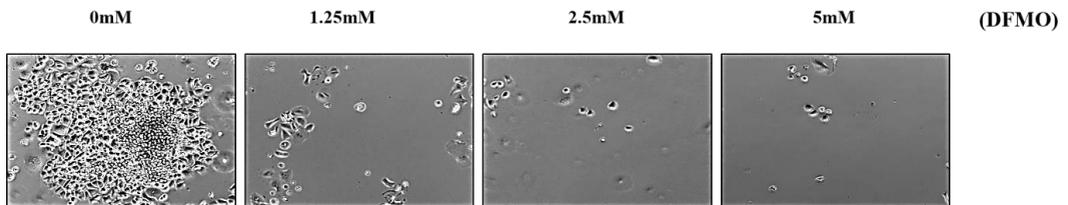
**Figure 3. DFMO inhibits breast cancer cell growth.**

(a) Cell viability was measured in cells treated with DFMO at 0, 1.25, 2.5, and 5mM by Cell Counting Kit-8 assay. (b) After treated with DFMO 2.5mM, growth for 7 days was also measured by Cell Counting Kit-8 assay. \* $p < 0.1$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

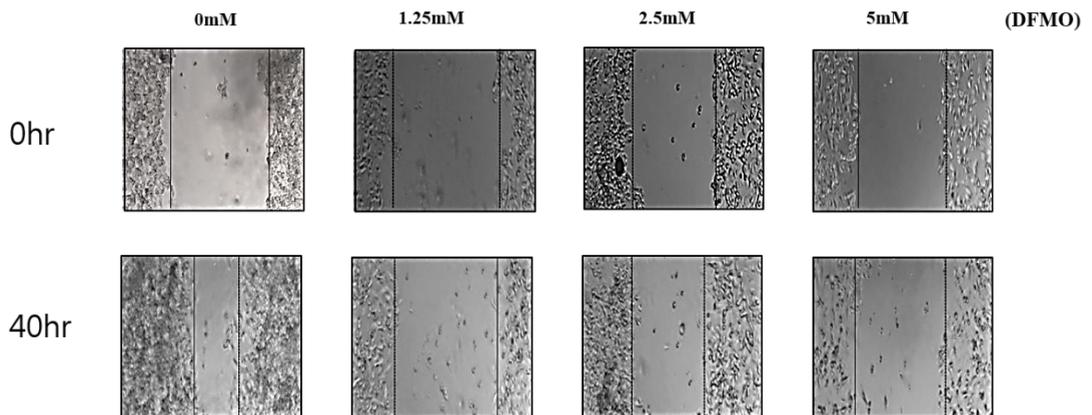
### 3. DFMO treatment decreased colony formation and cell migration ability.

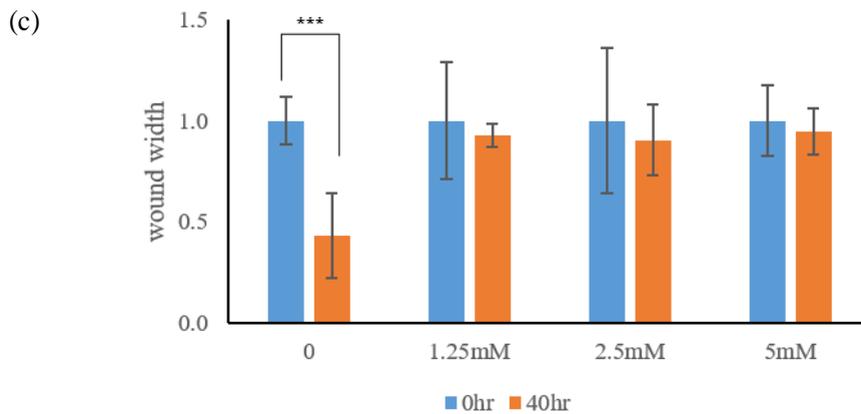
Unlike normal cells, when cancer cells grow excessively, they grow in multiple layers beyond the monolayer membrane, and form a colony. Thus, we conducted colony formation assays to determine if DFMO exhibited these properties in breast cancer cell lines. Fifteen days after DFMO treatment on SKBR3, colony formation efficiency was reduced from the lowest concentration of 1.25mM DFMO and is further rapidly at higher concentrations of DFMO (Figure 4a). Compared to the ability of each cell to migrate by wound healing assay, cells treated with 1.25, 2, and 5mM concentrations of DFMO healed at a slower rate than untreated cells and did not move for 40 hours after wounding (Figure 4b, c).

(a)



(b)



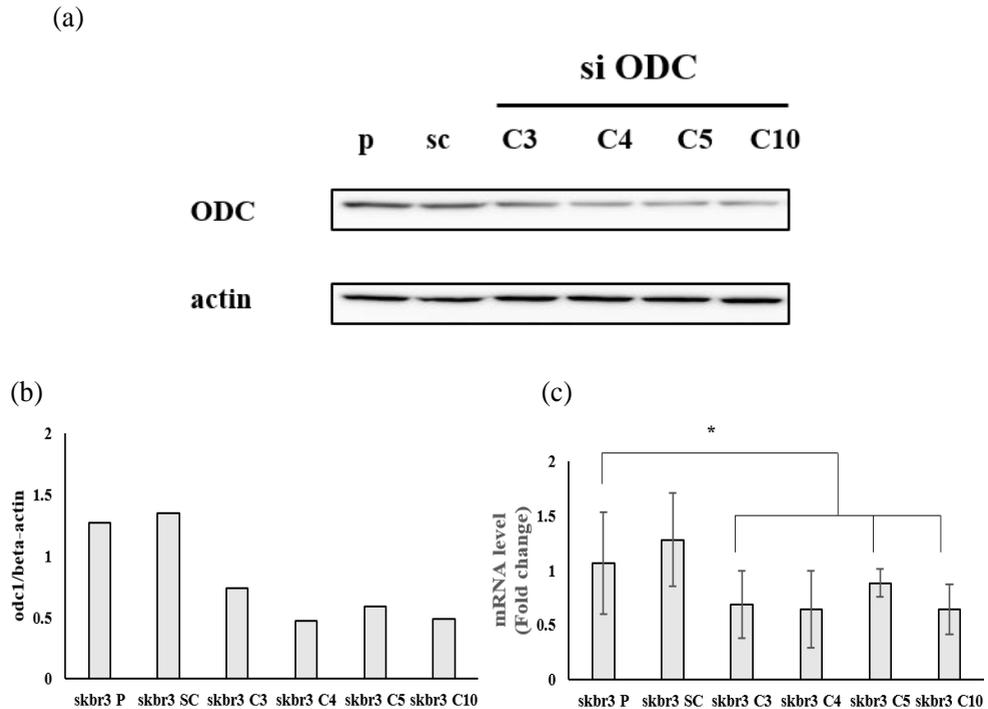


**Figure 4. DFMO inhibits cell colony formation and cell migration.**

(a) Colony formation ability of SKBR3 decreased from the lowest concentration of 1.25mM DFMO, which showed greater reduction at higher concentrations. (b)&(c) Comparison of the migration ability of each cell by wound healing assay. The migration rate of DFMO-treated SKBR3 from 1.25mM to 5mM was slower than that DFMO-untreated SKBR3. \* $p < 0.1$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

#### 4. Decreased ODC1 expression through siRNA transfection

We transfected siODC1 into a breast cancer cell, SKBR3 by lipofectamine to investigate the role of ODC1 in apoptosis and cell cycle arrest in breast cancer. We used a combination of two siRNAs to select the most effective combination. Combinations are that C3 are siODC 1+ siODC 4, C4 are siODC 1+ siODC 5, C5 are siODC 2+ siODC 3, and C10 are siODC 4+ siODC 5 (total 200pmol). We confirmed the reduction of ODC1 expression by siRNA transfection with western blot (Figure 5a). Our data showed that the expression of ODC1 was reduced, especially for siODC C4,10 (Figure 5b). The graph shows that reduced mRNA levels of ODC1 in siRNA combinations (Figure 5c).

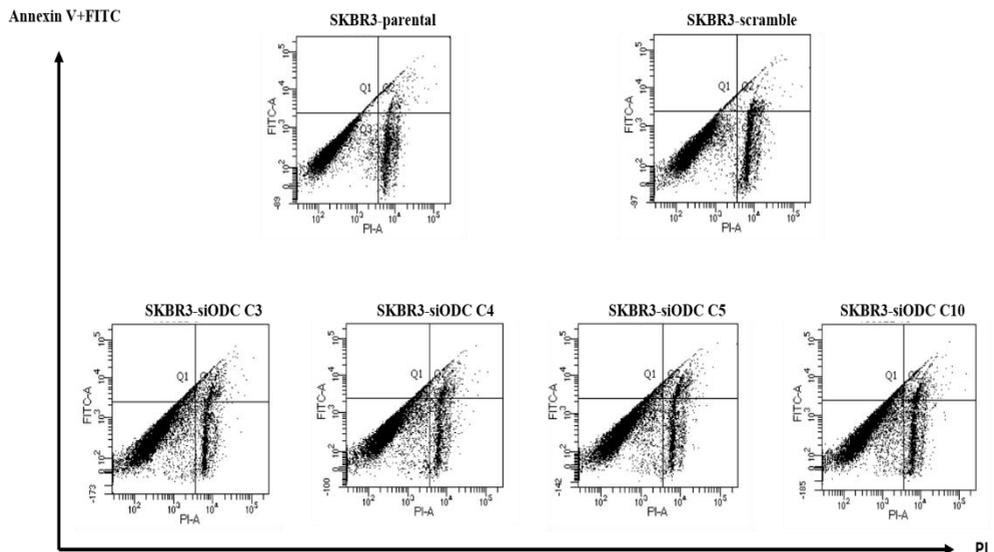


**Figure 5. Downregulation of ODC1 by siRNA** (a) ODC expression was analyzed by Western blot in SKBR3 cells expressing parental, scramble, and siODC. (b) ODC1 expression was reduced by half, especially si ODC1 C4 and 10, in a graph showing quantified data normalized with  $\beta$ -actin. (c) RT-PCR showed siODC1 transfection into SKBR3. The graph shows the reduced value compared to GAPDH by calculating the delta-delta CT value.

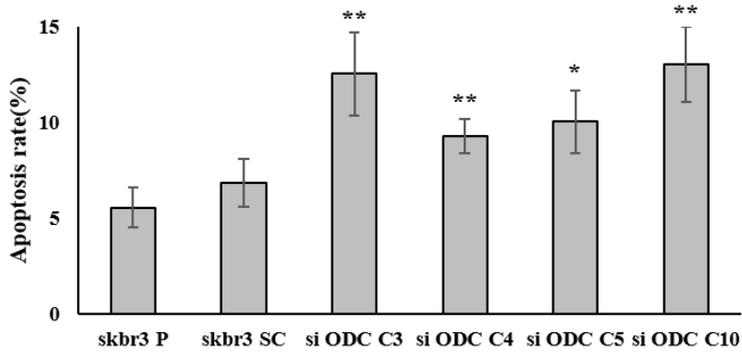
### 5. Reduced ODC1 expression in breast cancer cell induces apoptosis.

Apoptosis was measured by flow cytometry using Annexin V and PI staining of normal cells and cell with reduced ODC1 expression. Compared to parental and scramble cells, total apoptosis was more than doubled in ODC1 knocked down cells (Figure 6a). Normal cells with intact cell membranes were negative because PI and Annexin V + FITC were not stained. In early apoptosis, phosphatidylserine (PS) was released to outside, Annexin V + FITC was positive and PI was negative. In late apoptosis, dead cells were positive for PI and Annexin V + FITC. The apoptosis rate is the sum of early apoptosis corresponding to Q1 and late apoptosis or dead cells of Q2 (Figure 6b). If DNA damage does not recover above the repair level, not only caspase-3 and 9, but also cleaved PARP is activated and Bcl-2, which prevents apoptosis, is reduced, resulting in apoptosis. Western blot analysis showed that reduced ODC1 induced increased expression of cleaved PARP, caspase-3 and caspase-9, and suppressed expression of Bcl-2 (Figure 6c).

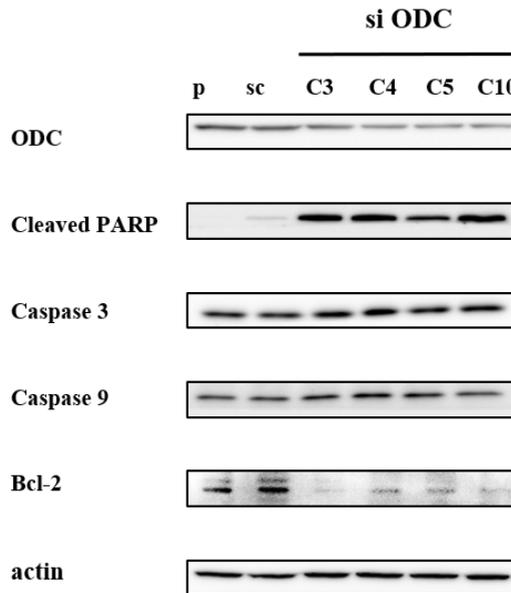
(a)



(b)



(c)

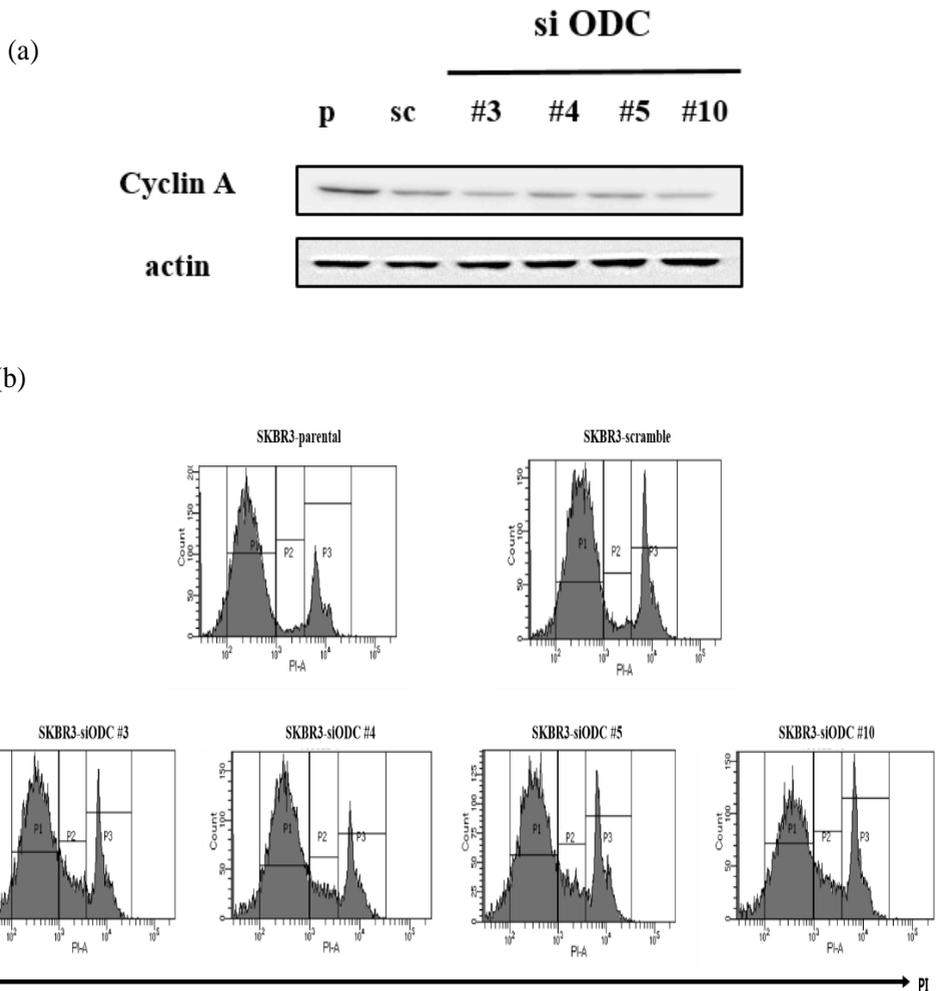


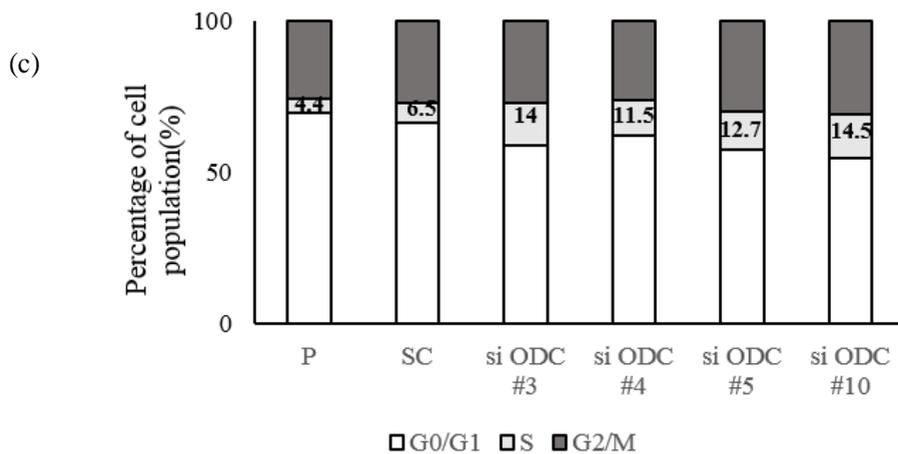
**Figure 6. Silencing of ODC1 expression by siODC1 induces apoptosis.**

(a) Apoptosis was induced when SKBR3 was transfected with siODC1. (b) The apoptosis rate, the sum of early apoptosis and late apoptosis or dead cells, was significantly increased after the siODC1 transfection. (c) Apoptosis induction was confirmed by the western blot. When ODC1 expression was decreased, expression of Cleaved PARP, Caspase-3, and Caspase-9 was increased, whereas Bcl-2 was reduced.

6. Downregulation of ODC1 in breast cancer cells induces cell cycle arrest at S phase.

We observed cyclin A by western blot in order to confirm that reduced ODC1 expression results in cell cycle arrest. Decreased expression of cyclin A stops DNA synthesis and replication. Therefore, the cell is accumulated in the S stage and can't proceed to the next step. Western blot analysis showed that reduced ODC1 induced decreased expression of cyclin A (Figure 7a). We analyzed cells stained with PI solution through flow cytometry and observed that cell cycle arrest at S phase was more than doubled in ODC1 knocked down cells, compared to parental and scramble cells (Figure 7 b, c).





**Figure 7. Silencing of ODC1 expression by siODC1 induces cell cycle arrest.**

(a) SKBR3 was transfected with siODC1 and it was confirmed by Western blot that cyclin A expression was decreased. (b) Cell cycle was analyzed by flow cytometry and cell cycle arrest occurred in S phase due to the decrease in ODC1 expression. (c) It is a graph of (b). P1 is G0 / G1, P2 is S, and P3 is G2 / M phase.

#### IV. DISCUSSION

Breast cancer is the most common cancer in women in the world. Like all tumors, breast cancer is caused by a regulatory disorder of intracellular signaling pathways associated with self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis.<sup>34,49 34,49</sup> For example, The estrogen steroid hormone receptor (ER) and human epidermal growth factor receptor 2 (HER2) are the mediators of two key pathways involved in breast cancer cell growth, metastasis, and invasion of other organs. ER is composed of six functional domains. Among them, activating function 1 (AF1) and activating function 2 (AF2) that regulate transcriptional activity are located in N-terminal A/B and hormone-binding domains, respectively.<sup>50</sup> When ER is activated by estrogen stimulation, it moves from the cytoplasm to the nucleus and binds to cis-regulatory DNA regions of genes, regulating the expression of other genes. ER stimulates the proliferation of breast cancer cells and increases cell division and DNA replication. This leads to mutation and cell cycle disruption, which increases tumorigenesis.<sup>51</sup> HER2 activates intracellular signals via PI3K/Akt/mTOR and MAPK/ERK pathways. When HER2 is overexpressed in breast cancer cells, the expression of Bcl-2 and Bcl-XL, which play an anti-apoptotic role, is up-regulated.<sup>52</sup> The mechanisms contributing to the development of breast cancer are complex, many studies are currently underway.

The intracellular concentration of polyamines, including putrescine, spermidine, and spermine, heavily influences cell growth and differentiation. Polyamines are present at high concentrations in exocrine and endocrine cells and exhibit the highest concentrations in insulin-producing beta cells. Inhibition of biosynthesis of polyamines by genetic engineering results in decreased levels of mass and differentiation of exocrine pancreas and beta cells.<sup>53</sup> Polyamines and polyamine-related enzymes have a role in enhancing the differentiation of stem cells and central transcription factors, and are involved in bone development. Polyamine, spermidine, and spermine are involved in the hypertrophy and terminal differentiation of chondrocytes.<sup>54</sup> In general, as the concentration of polyamine increases, cell growth rapidly increases. It is overexpressed in breast, colon, endometrial, and prostate cancers.<sup>30,32,55,56</sup> Overexpression of polyamines, including ODC1, promotes colony formation and migration of hepatocellular and colorectal cancer cells.<sup>57</sup>

Overexpression of ODC1 has been shown to contribute to the formation of tumors in mouse skin.<sup>58</sup>

DFMO is the best-known polyamine biosynthesis inhibitor. The L-enantiomer of DFMO forms an enzyme-inhibitor complex. DFMO functions by binding to the active sites of lys 69 and lys 360 of ODC1 after enzymatic decarboxylation and fluorine loss.<sup>42</sup> DFMO is a specific inhibitor of ODC1 activity accompanied by growth inhibition.<sup>59</sup> DFMO has a great effect in reducing glioma cell growth and invasion.<sup>60</sup> Depletion of cellular polyamines by DFMO substantially decrease colorectal cell growth.<sup>61</sup> DFMO attenuates proliferation in vascular smooth muscle cells and accumulated cells in the S phase of the cell cycle and reduces cyclin A expression.<sup>62</sup> DFMO also inhibits cell migration and invasion in neuroblastoma cell.<sup>63</sup> One clinical trial reported a combination of DFMO and PCV (procarbazine / lomustine / vincristine) was given to anaplastic glioma patients after radiation therapy. The study results indicate that survival was increased with combination of DFMO and PCV, compared with PCV alone.<sup>64</sup> In another clinical trial, two-year administration of DFMO in high-risk neuroblastoma patients, which account for 15% of childhood cancer deaths, has been shown to be effective in preventing relapse.<sup>65</sup>

In our study, the cells with high ODC1 expression show that cell growth is reduced from 3 days after the reaction with a low concentration of DFMO. The colony formation ability of cells treated with DFMO was lower than that of the control, and markedly decreased at higher concentrations. Migration of cells not treated with DFMO was very rapid, compared to cells treated with various concentrations of DFMO. However, in clinical trials investigating small cell lung cancer treatment, the use of DFMO alone or in combination with other inhibitors was shown to be minimally effective, and the toxicity issue was not resolved. Other concentrations of DFMO are effective, depending on the cancer type. So many studies are being conducted the issue, including toxicity studies, to enable the safe use DFMO in clinical practice.<sup>34,64,66</sup>

The relationship between ODC1 expression and apoptosis is still unknown. Apoptosis is a form of cell death that removes unneeded and dangerous cells from an organism, allowing tissue remodeling and homeostasis. Apoptosis destroys problematic DNA and cellular proteins, which are absorbed and killed by phagocytosis.<sup>67</sup> In some intestinal epithelial cell lines, depletion of polyamines either increases or decreases sensitivity to apoptosis depending on the nature of the apoptosis. Decreasing cellular polyamines

induces cell cycle arrest but, does not induce apoptosis in NIH3T3 cells.<sup>68</sup> Polyamine depletion has been shown to block apoptosis in rat intestinal epithelial cells by decreasing cytochrome c release, and reducing the activities of caspase-3 and 9.<sup>69</sup> Many reports indicate that apoptosis is induced when polyamines are reduced.<sup>33</sup> In the present study, when apoptosis is measured by flow cytometry after staining cells with Annexin V and PI, apoptosis is increased in cells with ODC1 downregulated by siRNA compared to parental and scramble cells. In addition, siODC1 C3, 4 increased the expression of caspase-3, 9, and cleaved PARP. In contrast, anti-apoptotic Bcl-2, which induces anti-apoptosis signaling, was decreased. When DNA damage does not recover beyond the repair level, p53 activates Bax expression and Bax induces cytochrome c release, which in turn induces caspase-3, and 9 in the mitochondrial pathway.<sup>70-72</sup> Anti-apoptotic Bcl-2 proteins tightly bind all BH3 proteins to inhibit apoptosis, and Bax can heterodimerize with Bcl-2 to provoke apoptosis.<sup>73</sup> Poly [ADP-ribose] polymerase-1 (PARP-1) is a nuclear enzyme that has a role in protecting the genome from DNA damage. When PARP-1 is activated, apoptosis-inducing factor translocate from the mitochondria to the nucleus,<sup>74</sup> Once PARP is cleaved by caspases, apoptosis is induced.<sup>75</sup> Our results confirmed that inhibiting ODC1 in breast cancer cell resulted in cell death due to apoptosis.

Polyamines are essential for cell growth and proliferation. The transcription of ODC1 is activated by c-MYC and linked to the cell cycle and expressed in the G1 / S phase.<sup>76</sup> DFMO induces G1 phase arrest of human gastric cancer cell (MKN45) for up to 72 hours through the expression of p21 and phosphorylation of stat1.<sup>77</sup> DFMO acts by blocking ODC1 activity, and accumulates the cells in S phase of the cell cycle in vascular smooth muscle cells.<sup>62</sup> Polyamine biosynthesis changes peak periodically as enzyme activity is regulated during transcription, translation, and post-translation during the cell cycle. When polyamines do not increase normally during cell proliferation, DNA replication is negatively affected. The most sensitive cell cycle is the S phase.<sup>78</sup> Decreased cyclin A expression prevents DNA synthesis and slows the rate at which cells accumulate in the S phase and cross over to G2 / M.<sup>62</sup> The results of the current study suggest that reduced ODC1 expression arrests breast cancer cell cycle in the S phase by decreasing the cyclin A expression. When confirming the cell cycle by flow cytometry after staining with PI, cell cycle arrest also occurs in S phase at cells reduced ODC1 expression.

## V. CONCLUSION

The purpose of this study was to characterize the role of ODC1 in breast cancer. We showed that low concentration of DFMO inhibits the growth of breast cancer cell with high ODC1 expression. But other cells that had low ODC1 expression were less sensitive to high DFMO concentration. Our study also demonstrated that ODC1 depletion not only increased apoptosis, but also induced cell cycle arrest of the breast cancer cell. These results suggest that ODC1 may be a target for the treatment of breast cancer. Developing new drugs that more effectively inhibit ODC1 would be helpful to treat breast cancer.

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ABSTRACT(IN KOREAN)

## 유방암에서의 Ornithine decarboxylase 1(ODC1) 의 역할 규명

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신 윤 진

Ornithine decarboxylase(ODC1)는 폴리아민 생합성에서 푸트레신의 생산에 관여하는 주요 효소로 핵산 대사, 세포 성장, 분화 및 세포주기에 영향을 미치는 것으로 알려져있다. 증가 된 ODC1은 발암과 관련이 있으며 침습 및 신생 혈관 생성에 필수적이다. 몇몇 선행연구에서는 ODC1의 감소가 암 세포의 증식 및 암의 전이를 억제한다고 밝혀져 있지만 유방암 세포에서 ODC1이 세포 성장, 증식 및 침습을 어떻게 조절하는지에 대한 많은 연구가 필요하다. 본 연구에서 Pyridoxal phosphate 대신에 ODC1에 결합함으로써 ODC1의 작용을 억제하는 Difluoromethylornithine (DFMO)의 처리로 유방암 세포 성장, 세포 이동 및 세포 콜로니 형성 능력이 현저히 감소됨을 확인하였다. 또한 실험을 통해 ODC1의 발현감소는 세포사멸을 유도하고 세포주기의 S 단계에서 세포의 축적 결과를 확인하였다. 유방암 세포에서 ODC1의 발현감소는 cleaved PARP, caspase3,9 의 발현을 촉진하고 미토콘드리아 경로에서 Bcl-2의 발현을 감소시켰다. 따라서 이 연구의 결과는 ODC1을 포함하는 폴리아민 경로가 암 세포주기에서 중요한 역할을 하고 ODC1의 발현을 억제했을 때 세포주기 순환이 막혀 세포사멸이 일어나 암 세포의 증식 및 전이를 억제함을 제시한다. 이에 ODC1에 대한 연구 가능성을 열었고 앞으로 ODC1이 유방암 치료를 위한 유망한 표적임을 증명하는 더 많은 연구가 필요하다.

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Key word : 유방암, ODC1, DFMO , 세포주기, 세포증식