# The Regulation of OCT4 Ubiquitination by E3 Ligase CHIP that Effects on Tumorigenesis of Breast Cancer

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# The Regulation of OCT4 Ubiquitination by E3 Ligase CHIP that Effects on Tumorigenesis of Breast Cancer

**Directed by Professor Kyung-Hee Chun**

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# This certifies that the Doctoral Dissertation of Yunhee Cho is approved.

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**Yunhee Cho**

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# ABSTRACT

## The regulation of OCT4 ubiquitination by E3 ligase CHIP that effects on tumorigenesis of Breast Cancer

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Breast cancer has been considered to be hierarchically organized and it contains cancer stem cells (CSCs), which do self-renewal and relapse after therapy. Therefore, characterization and targeting CSCs are an issue recently. Because the role of protein stability and homeostasis against initiation and maintenance of CSCs was not elucidated, after preparation of mammospheres using breast cancer MDA-MB231 cells and MCF-7 cells, I performed DNA microarray analysis and observed that the expression of CHIP E3 ubiquitin ligase was down-regulated in both cell-made mammospheres. Ablation of CHIP increased mammosphere formation, whereas

overexpression of CHIP reversed these effects. I searched the interactomes by mass-spectrum analysis and found that CHIP directly interacted with OCT4. Overexpression of CHIP reduced OCT4 stability by proteosomal degradation. K30A chaperone-binding mutant CHIP showed less interaction with OCT4, suggesting that CHIP interacts with OCT4 by chaperone-dependent manner. Whereas CHIP induced ubiquitination of OCT4, H260Q catalytic mutant CHIP couldn't effect it. Interestingly, I determined 284 lysine of OCT4 is an ubiquitination site by CHIP. Overexpression of CHIP couldn't degrade K284R mutant OCT4. Overexpression of CHIP reduced cell proliferation and side population of breast cancer cells, however co-overexpression of K284R mutant OCT4 and CHIP didn't reduce them. Moreover, only 1,000 cells with CHIP depleted or OCT4 overexpressed could generate breast tumors and metastasis in xenografted mice model. Breast cancer patients with low-expressed CHIP E3 ligase have poor survival probability. Taken together, ubiquitination of OCT4 by CHIP plays a role in breast tumorigenesis and I propose that regulation of OCT4 stability could be a potent approach in breast cancer therapy.

Key word: CHIP, Ubiquitination, OCT4, Tumorigenesis, Breast Cancer

# The regulation of OCT4 ubiquitination by E3 ligase CHIP that effect on tumorigenesis of Breast Cancer

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## I. INTRODUTION

Breast cancer is the second leading cause of cancer death in women.[1] According to the development in the therapeutic modalities, the 5-year survival rate of breast cancer patients has been improved substantially, but the recurrence and the subsequent drug resistance for breast cancer seem to be main lethal reasons.[2] A hypothesis for the lethality of breast cancer has been proposed to explain by "cancer stem-cell (CSC) model", which explains that malignancies arise from a small subset of stem cell-like cancer cells.[3] It was also demonstrated that cancerous tumors contain a much higher proportion of CSCs than benign tumors and that the malignancy of tumors is correlated with the proportion of CSCs. As like stem cell regulatory

pathways, Wnt/β-catenin, Notch, Hedgehog and Bone Morphogenetic protein (BMP) pathways play important roles in regulation of CSCs.[4] Potential CSC markers include cell surface receptors, such as CD133, CD44 and EpCAM, high enzymatic activities, such as aldehyde dehydrogenase (ALDH), and transcriptional factors, such as SRY-box 2 (Sox2), Nanog homeobox (Nanog), and POU class 5 homeobox 1 (POU5F1 or Oct-3/4).[5] Octamer-binding transcription factor 4, *Oct4*, also known as *POU5F1*, is a transcription factor and a critical regulator of stemness for embryonic stem cell maintenance.[6,7] In cancer, several evidence shoswed that OCT4 regulates the tumorigenesis through inducing angiogenesis and EMT, and tumor growth.[8] Most studies about OCT4 showed the down- or up-stream regulatory mechanism in transcription levels.[9] Recently, protein modification of OCT4 is reported that relates with transactivation[10] or DNA binding activity,[11] however, other protein modifications of OCT4 are unclear.

However, the existence of CSCs has been controversial until now[12], and there are still poor evidences how CSCs initiate and maintain in tumors. I started this study to figure out the characterization of breast CSCs by mammosphere culture assay. Because CSCs are likely to be only a small population in cancer cells, the method for identifying and enriching them is significant so the mammosphere technique has been developed.[13] Dontu et al. demonstrated that the serum-free nonadherent culture technique could enrich the breast stem cells within a population of primary human mammary epithelial cells.[14] It was applied to enrich the CSCs from breast cancer cells.

I prepared mammospheres by MCF7 and MDA-MB231 breast cancer cells, and performed DNA microarray analysis.

Among the results, I was interested in the expression levels of E3 ubiquitin ligases, because the role of protein stability and homeostasis against initiation and maintenance of CSCs was not elucidated, yet. I found out the significant down-regulation of CHIP, an E3 ubiquitin ligase in both cell-made mammospheres. The carboxy-terminus of Hsc70 interacting protein (CHIP) is known to function as a chaperone associated E3 ligase for several proteins and regulates a variety of physiological processes.[15] CHIP is involved in diverse cellular processes, such as protein trafficking, degradation, signaling, transcription, and apoptosis. Accumulating evidences indicate that CHIP plays an important role in cancers, neurological disorders, cardiac diseases and bone metabolism. In cancers, CHIP seems to be a dispute regarding the oncogenic or tumor suppressive role. It was reported that tumor growth and metastasis were negatively correlated to CHIP levels in breast tumor bearing nude mice.[16] CHIP also seems to regulate the levels of a number of well-known oncogenic proteins like TRAF2, NF-ƙB, PTK6 and MIF.[17-19] Further supporting its role as a tumor suppressor CHIP has been reported to degrade a number of other critical oncoproteins such as AKT, MYC, and HIF-1α in various cancers.[20-22] However, on contrary to the above mentioned reports, in a number of other publications CHIP can function as an oncogene. CHIP promoted the ubiquitination and degradation of FoxO1 and antagonized FoxO1-mediated proapoptotic program and enhanced survival and proliferation.[23] Similarly, PTEN was shown to be a target

for CHIP mediated ubiquitination and degradation in prostate cancer cells.[24] Clearly, the role of CHIP in tumors is not straight forward, yet.

In this study, I demonstrated how CSCs maintain their population in breast cancer cell lines. Especially, CSCs have down-regulated CHIP E3 ligase and regulate post-translational modification of OCT4, which is a stemness marker, because OCT4 level is important in the determination of the mouse embryonic stem cell fate and cancer tumorigenesis. Therefore, I investigated how CSCs regulate OCT4 protein expression level and breast tumorigenesis by down-regulation of CHIP E3 ligase.

# II. MATERIALS AND METHODS

## 1. Cell culture and transfection

The human breast cancer cell lines MDA-MB-231 and MCF7 were obtained from ATCC (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (Invitrogen, Carlsbad, CA, USA) at 37℃ in an atmosphere of 5% $CO_2$. Transfection with CHIP, WT and mutant, expression vectors[25] and Oct4 expression vector[26] as well as with CHIP and Oct4 siRNA were performed with Lipofectamine 2000 and Lipofectamine RNAiMAX reagent (Invitrogen) respectively, according to the manufacturer's instruction. *CHIP* siRNA #1 (5′-CCCAAGUUCUGCUGUUGGACU-3′), *CHIP* siRNA #2 (5′-GAAGAGGAAGAAGCGAGACAU-3′), *CHIP* siRNA #3 (5′-GCAGUCUGUGAAGGCGCACUU-3′), and *OCT4* siRNA (5′-UUAAGUUCUUCAUUCACUAAG-3′) were purchased from COSMOGENETECH (GenePharma Co, Shanghai, China). Cells were harvested two days after the transfection for use in subsequent experiments.

## 2. Mammosphere culture

Cells (density, 1,000 cells/ml) were grown in ultra-low attachment plates (Corning Costar, Acton, MA, USA) containing mammary epithelium basal medium (Lonza, Basel, Switzerland) supplemented with B27 (Gibco, Grand island, NY, USA), 20 ng/ml EGF, and 20 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA). After culturing

for 15 days, mammospheres with diameters of >50 μm were counted using a microscopy.

### 3. Mutagenesis and generation of stable cell lines

Point mutation of lysine to arginine at position 284 (K284R) in OCT4 was induced by performing site-directed mutagenesis using EZchange™ site-directed mutagenesis kit (Enzynomics, Seoul, Korea), according to the manufacturer's instructions. Primers used are shown in Table 1. The shRNA-expressing lentiviral vectors for CHIP and OCT4 depletion ($CHIP_{KD}$ and $OCT4_{KD}$ cells) that targeted the 3′-UTR of the encoding genes were purchased from Sigma (*CHIP*: TRCN0000007525, TRCN0000007526, and TRCN0000007527; *OCT4*: TRCN0000235522, St. Louis, MO, USA). Lentivirus particles were generated using VSVG, RSV-REV, and PMDLg/pPRE vectors, in which 293FT cells were cotransfected with the shRNA-expressing lentiviral vectors. The 293FT cells were transfected using Lipofectamine 2000 following the manufacture's instruction. 48 hr after transfection, the 293FT cells cultured media was filtered using a 0.45 μm filter. Lentivirus infections were performed in media of MDA-MB-231 and MCF7 cells. The infected cells were selected for stable depletion or overexpression using puromycin (Sigma-Aldrich).

### 4. Total RNA isolation and reverse transcription-polymerase chain reaction

RNA was isolated using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a reverse transcription system (TOYOBO, Tokyo, Japan) and primers listed in Table 1. PCR was performed using instructions given in Ex-Taq (TaKaRa, Kyoto, Japan) manual.

**5. Microarray analysis**

Gene expression in the examined cell lines was analyzed using high-density oligonucleotide microarrays containing 20,889 transcripts (HG-U133 Plus 2.0; Affymetrix). Target preparation and microarray processing were performed as described in Affymetrix GeneChip expression analysis manual. GeneChip analysis was performed using Affymetrix GeneChip manual with Microarray Analysis Suite 5.0, Data mining Tool 2.0, and Microarray Database software.

**6. Luciferase assay**

OCT4 reporter assay was performed using *OCT4* promoter construct phOCT4-Luc.[27] MCF7 cells were transfected with the CHIP expression vector or *CHIP* siRNA, and β-galactosidase expression vector was used for normalization. After 48 hr, luciferase activity was measured using a luciferase assay system (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions.

**7. Side population analysis**

Cells were transfected with the indicated vectors or siRNAs and were harvested after 48 hr. Next, $1 \times 10^6$ cells were incubated in 1 ml suspension medium (HBSS, 2% FBS, and 10 mM HEPES) containing 5 μg/ml Hoechst 33342 dye (Thermo Fisher Scientific, San Jose, CA, USA) and 50 μM verapamil (Sigma Aldrich) at 37℃ for 60 min. The cells were then washed three times with a cold suspension medium and were treated with 2 μg/ml PI solution. A minimum of 20,000 events/sample were collected using FACSDiva and Cell Quest applications (BD Bioscience, San Jose, CA, USA).

**8. Cell viability analysis**

Cells were grown in 96-well culture plates and were transfected with *CHIP* and *OCT4* siRNAs or CHIP and WT OCT4 and OCT4_K284 expression vectors. After 48 h, WST solution (Daeil, Soeul, Korea) was added to each well. After 1–3 hr of incubation, absorbance was measured using ELISA reader at a test wavelength of 450 nm.

**9. Ubiquitination assay**

Ubiquitination assay was performed under denaturing condition, as described previously.[28] Briefly, cells were lysed in 6 M guanidinium-HCl buffer (pH 8) containing 5 mM N-ethylmaleimide, (Sigma-Aldrich) to prevent deubiquitination. OCT4 was purified by incubating with OCT4 specific antibody (sc-5279; Santa Cruz, Dallas, TX, USA), washed, eluted in sample buffer containing 200 mM imidazole and 10 mM β-mercaptoethanol (Sigma-Aldrich), and then analyzed by Western blot.

Western blotting was performed using the HRP-conjugated anti-Ub antibody (BML-PW0150; ENZO, Lausen, Switzerland).

**10. Immunoprecipitation and western blotting**

Cell lysates were incubated with the following antibodies: mouse IgG (sc-3877; Santa Cruz), anti-FLAG antibody (F1804; Sigma Aldrich), and anti-OCT4 antibody (sc-5279; Santa Cruz, Dallas). Immunoprecipitation was performed, as described previously.[26]

Western blotting was performed using the following antibodies: anti-GAPDH (sc-25778), anti-CHIP (sc-66830), anti-Nanog (sc-33759), anti-SOX2 (sc-20088), and anti-OCT4 antibodies (all purchased from Santa Cruz, Dallas, TX, USA); anti-FLAG antibody (Sigma Aldrich); and HRP-conjugated anti-Ub antibody (BML-PW0150; ENZO). Proteins of interest were detected using an ECL solution (Bio-Rad, Hercules, CA, USA) with LAS-3000 detector (Fujifilm, Tokyo, Japan) according to the manufacturer's directions.

**11. Immunohistochemistry**

Xenografted tumors were obtained and fixed in 4% paraformaldehyde (Biosesang, Seoul, Korea). The fixed tumors were embedded in paraffin blocks and were sliced into 0.4-μm-thick sections. CHIP and OCT4 levels in the xenografted tumors were immunohistochemically detected using Vectastain ABC kit and DAB

substrate kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's directions.

**12. Mass spectrometry and network analysis**

For identification of CHIP-interacting proteins in MDA-MB231 cells, CHIP immunoprecipitation eluates were separated by SDS-PAGE and subjected to in-gel tryptic digestion following the general protocol.[29] Extracted peptides were suspended in 0.1% FA in water, loaded onto an EASY-Spray C18 column (75 μm×50 cm, 2 μm) and separated with a 2%–35% gradient of 0.1% FA in ACN for 65 min at a flow rate of 300 nL/min. MS spectra were recorded on a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) interfaced with a nano-ultra-HPLC system (Easy-nLC1000; Thermo Fisher Scientific). Collected MS/MS raw files were converted to mzXML files using the Trans-Proteomic Pipeline (version 4.4) and analyzed using the Sequest (version 27) algorithm in the SORCERER (Sage-N Research, Milpitas, CA, USA) platform. Protein database search was performed using the Uniprot human database (version 2016.06, 313072 entries). Full tryptic specificity and up to two missed cleavage sites were allowed. Mass tolerances for precursor ions and fragment ions were set to 10 ppm and 1 Da, respectively. Fixed modification for carbamidomethyl-cysteine (+57.0215 Da) and variable modifications for methionine oxidation (+15.9949 Da) were used. All proteins with a ProteinProphet probability of $\geq$ 95% with minimum two peptides and a PeptideProphet probability of $\geq$ 90%,

peptide FDR ≤ 0.3% were identified using Scaffold (version 4.3.2; Proteome Software, Portland, OR, USA).

For ubiquitination site mapping of OCT4, purified OCT4 protein from HEK293 cell line was separated by SDS-PAGE and subjected to in-gel digestion with trypsin/LysC mix (Promega). Collected MS/MS raw files were analyzed using the Proteome Discoverer (version 1.4). Di-glycine modification (GG, +114.043 Da) on the lysine and missed tryptic cleavage at the modified site were used. Human database and other search parameter were set with the same values as above.

Network analysis of selected CHIP-interacting proteins was performed by using the Ingenuity Pathway Analysis (IPA) software (Ingenuity System Inc, USA). Protein interaction networks functionally associated with OCT4 and CHIP were merged to generate a protein interaction network constituted by OCT4 and CHIP.

**13. Animal experiments**

All animal experiments were approved by the Institutional Review Board of the Yonsei University College of Medicine and were performed in specific pathogen-free facilities according to the university's guidelines for the Care and Use of Laboratory Animals (2015-0376). Xenografted mice and mice showing lung metastasis were generated, as described previously.[30, 31] 8-week old female Balb/c-nude mice (Orient, Seongnam, Korea) were subcutaneously inoculated with MDA-MB-231 cells ($1 \times 10^3$, $1 \times 10^4$ and $1 \times 10^5$ cells). For the lung metastasis, $1 \times 10^6$ cells were injected into 8-week old female Balb/c-nude mice (five mice per group) through the lateral tail vein.

4 or 6 weeks after, mice were sacrificed with an overdose of anesthesia. Xenografed tumors and the number of metastatic nodules on the surface of the lung were counted under the surgical microscope.

**14. Kaplan–Meier analysis**

Kaplan–Meier analysis of survival curve was performed using http://kmplot.com/analysis and two gene symbols *STUB1* (Affy ID: 217934_x_at) and *POU5F1* (Affy ID: 208286_x_at) for patients with breast cancer.

**15. Statistical analysis**

Tumors were isolated per mouse and were analyzed to determine mean tumor volume per mouse. Unpaired *t*-tests were used to analyze mean tumor volume in the xenografted mice. Statistical analyses were performed using Student's *t*-test with GraphPad Prism software (version 6; GraphPad Software Inc., La Jolla, CA, USA). Data were considered statistically significant at $p < 0.05$.

**Table 1. Primer lists and sequences for mutagenesis and RT-PCR**

| Primer | Sequence (5' to 3') | Applications |
|---|---|---|
| **CHIP_For** | CGACTACCTGTGTGGCAAGA | CHIP RT-PCR |
| **CHIP_Rev** | CAAGTTGGGGATGAGCTGTT | |
| **Nanog_For** | ACCTTCCAATGTGGAGCAAC | Nanog RT-PCR |
| **Nanog_Rev** | GAATTTGGCTGGAACTGCAT | |
| **Sox2_For** | AAAACAGCCCGGACCGCGTC | Sox2 RT-PCR |
| **Sox2_Rev** | CTCGTCGATGAACGGCCGCT | |
| **Oct4_For** | CTCACCCTGGGGGTTCTATT | Oct4 RT-PCR |
| **Oct4_Rev** | CTGGTTCGCTTTCTCTTTCG | |
| **GAPDH_For** | GGCTGCTTTTAACTCTGGTA | GAPDH RT-PCR |
| **GAPDH_Rev** | ACTTGATTTTGGAGGGATCT | |
| **Oct4_K284R_For** | AGCGAGGCAAGCGATCAAGCAG | Oct4 K284R mutagenesis |
| **Oct4_K284R_Rev** | GGCGCCGGTTACAGAACCACAC | |

# III. RESULTS

## 1. Down regulation of CHIP E3 ligase in mammospheres of MCF7 and MDA-MB-231 breast cancer cells

To identify the alterative expression of E3 ligases in mammosphere culture compared to normal culture condition, I performed a DNA microarray analysis (Fig. 1A). Several E3 ligases were upregulated or downregulated in mammosphere. However, I focused on the downregulated E3 ligases, which might degrade oncoproteins. I found that CHIP was downregulated in serial passage cultures of mammosphere, and rescued in re-adherent culture (Fig. 1B). Next, I generated the CHIP overexpressed or depleted stable lines, $CHIP^{over}$ or $CHIP_{KD}$ (Fig. 1C). I analyzed the mammosphere forming ability in the indicated stable lines. The mammosphere size and number were decreased in $CHIP^{over}$ lines, and increased in $CHIP_{KD}$ lines (Fig. 1D and E).

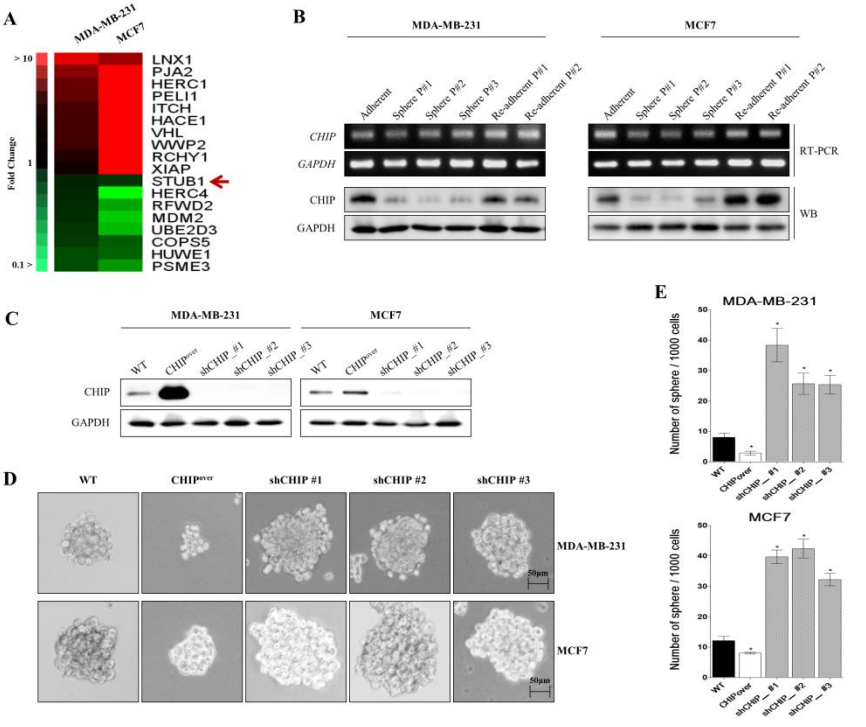



**Figure 1. The expression of CHIP influences mammosphere forming ability.** In MDA-MB-231 and MCF7 cells, (A) Alterative expression of E3 ligases was measured by a microarray analysis with mammosphere culture, compared to normal culture condition. (B) The expression level of CHIP was detected by RT-PCR (upper) and western blot (lower), respectively. GAPDH was used as a loading control. (C) The cells were infected by lenti-viral system for generation of the indicated stable cell lines. Overexpression and depletion of CHIP were confirmed by western blot. (D) Mammosphere forming ability of indicated stable cell lines was measured under sphere forming conditions for 15 days. (E) The number of spheres was quantified in the experiments (D). Data are presented as mean ± SD ($n$ = 3). Significant differences

are indicated by an asterisk (* $p<0.05$), and $p$ values were calculated using the Student's $t$ test.

## 2. CHIP E3 ligase interacts with OCT4 and induces its proteosomal degradation

Next, I identified CHIP interacting proteins with FLAG-tagged CHIP-expressing MDA-MB-231 by mass spectrometry (Fig. 2A). Among the total identified proteins, I selected CHIP specific proteins excluding IgG specific proteins and keratin proteins, and listed the developmental related proteins in interactomes of CHIP (Table 2). Interestingly, I found the OCT4 that is critical for stemness. Using the ingenuity pathway analysis, IPA, the interaction network was shown that CHIP correlated with OCT4 (Fig. 2B). Furthermore, breast cancer patients with highly-expressed *Oct4* showed the poor post progression survival probability (Fig. 2C). The post progression survival was defined that was recorded from progression until death, and correlated with the metastasis[32] and most progressive status.[33]

To test whether CHIP regulates the stability of the OCT4, I determined the expression of OCT4 in the absence of CHIP. Compared to scRNA transfected cells, I observed that the OCT4 levels were upregulated in the CHIP depleted cells, independent of its mRNA level (Fig. 2D). Furthermore, CHIP overexpressed cells showed that OCT4 was downregulated, independent of its mRNA level, and OCT4 protein levels were rescued in the proteasome inhibitor MG132 treated cells (Fig. 2E). In the increasing amount CHIP overexpressed and protein synthesis inhibitor cycloheximide, CHX, treated cells, OCT4 levels were decreased that reversely correlated with transfected CHIP amount (Fig. 2F). These data suggest that CHIP regulates the OCT4 stability through the proteosomal degradation.

**Table 2. Developments related genes on CHIP interactomes**

| Accession Number | Gene Symbol |
|---|---|
| P24752 | ACAT1 |
| P60709 | ACTB |
| P49189 | ALDH9A1 |
| P62330 | ARF6 |
| P27708 | CAD |
| B2RCM3 | CAPN2 |
| P60953 | CDC42 |
| B4DP52 | DDX39B |
| P11413-3 | G6PD |
| Q14103-2 | HNRNPD |
| P11717 | IGF2R |
| P00338-3 | LDHA |
| P09382 | LGALS1 |
| P43034 | PAFAH1B1 |
| P14618 | PKM |
| Q01860 | POU5F1 |
| P25788-2 | PSMA3 |
| P25789 | PSMA4 |
| P61586 | RHOA |
| Q93045-2 | STMN2 |
| P08670 | Vimentin |
| P62258-2 | YWHAE |
| P27348 | YWHAQ |

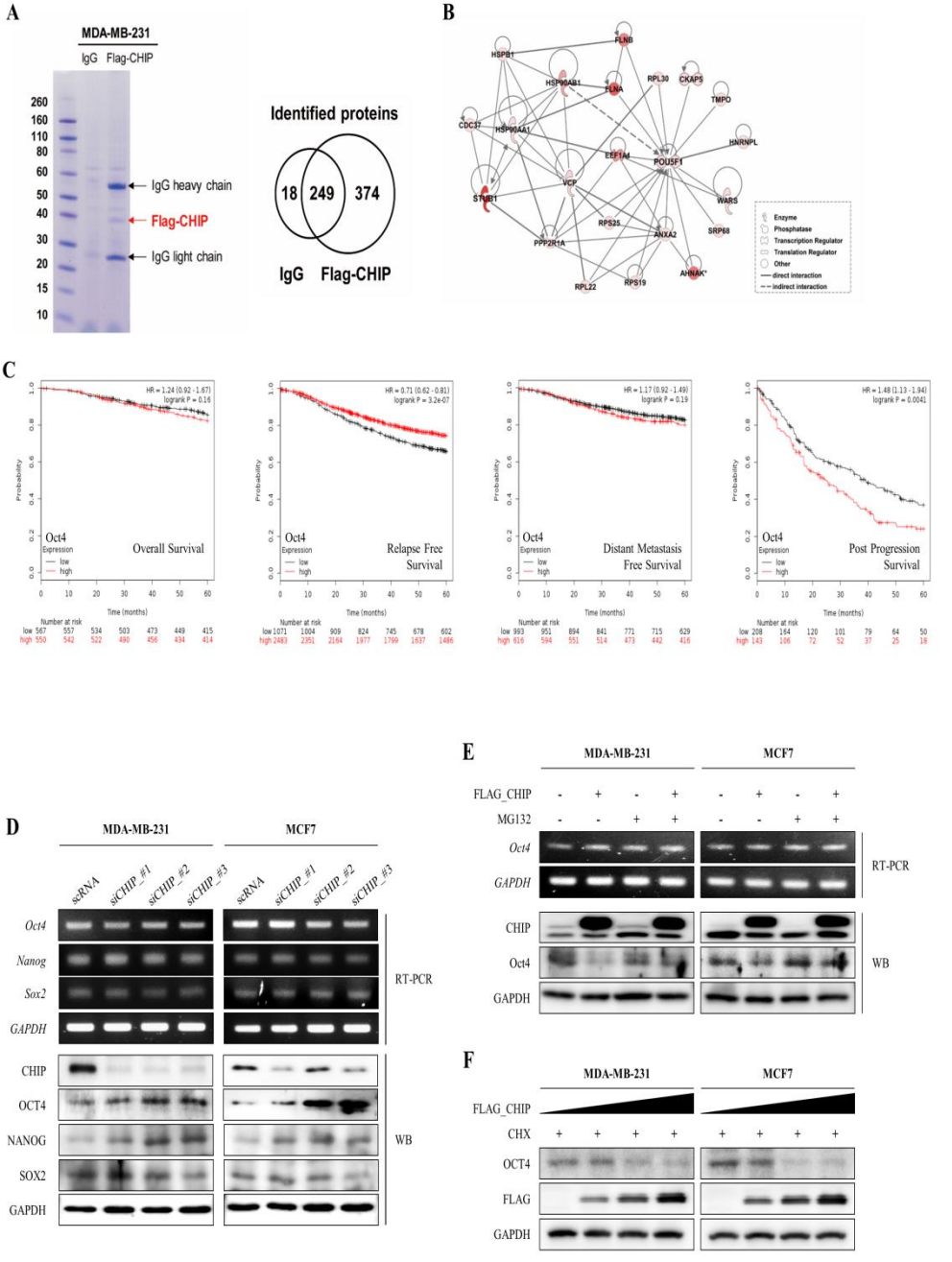


**Figure 2. The E3 ligase, CHIP regulates the OCT4 protein stability via the proteosomal degradation.** (A) Coomassie blue-stained gel of affinity-purified CHIP complex in MDA-MB-231 cells. The Venn diagram indicated the relationships between the identified proteins of each immunoprecipitation by mass spectrometry. (B) Molecular interaction network with CHIP and OCT4 using Ingenuity Pathway Analysis, IPA. (C) Overall, relapse free, distant metastasis and post progression survival of breast cancer patients were analyzed with *Oct4* expression level using Kaplan-Meier plot analysis. (D) MDA-MB-231 and MCF7 cells were transfected with scramble and CHIP siRNA. After 48 hr, mRNA (upper) and protein (lower) levels of indicated genes were detected by RT-PCR and western blot, respectively. (E) The cells were transfected with empty and FLAG_CHIP expression vector for 40 hr, and treated with 20 μM MG132 for an additional 8 hr. mRNA (upper) and protein (lower) levels of indicated genes were detected by RT-PCR and western blot, respectively. (F) The cells were transfected with increasing amount FLAG_CHIP for 40 hr, and treated with 20 μM CHX for an additional 8 hr. The protein levels of OCT4 and FLAG_CHIP were detected by western blot. GAPDH was used as a loading control.

### 3. CHIP E3 ligase interacts with OCT4 and regulates the OCT4 that related with chaperon

Next, I investigated the interaction of CHIP with OCT4. The interaction between CHIP with OCT4 was detected in MDA-MB-231 cells (Fig. 3A). Notably, the interactions between OCT4 with wild-type CHIP, and E3 ligase functional negative mutant H260Q, were detected, whereas the interaction between OCT4 with tetratricopeptide repeat, TPR, domain mutated CHIP, K30A, was not (Fig. 3B). The OCT4 levels were decreased in the only wild-type CHIP overexpressed cells, independent of its mRNA level (Fig. 3C). I found the OCT4 levels in the HSP90 inhibitor, 17-AAG, treated cells, because the interaction between OCT4 with K30A mutant CHIP was not detected, and CHIP is an HSP70 and HSP90 complex interacting E3 ligase at TPR domain.[34] The OCT4 protein levels were decreased in 17-AAG treated cells (Fig. 3D), and rescued in the CHIP depleted cells (Fig. 3E). Interestingly, CHIP-mediated down-regulation of OCT4 was detected either in the absence or presence of 17-AAG (Fig. 3C and E). These data indicate that the CHIP E3 ligase regulates the OCT4 stability, and it is related with chaperon.

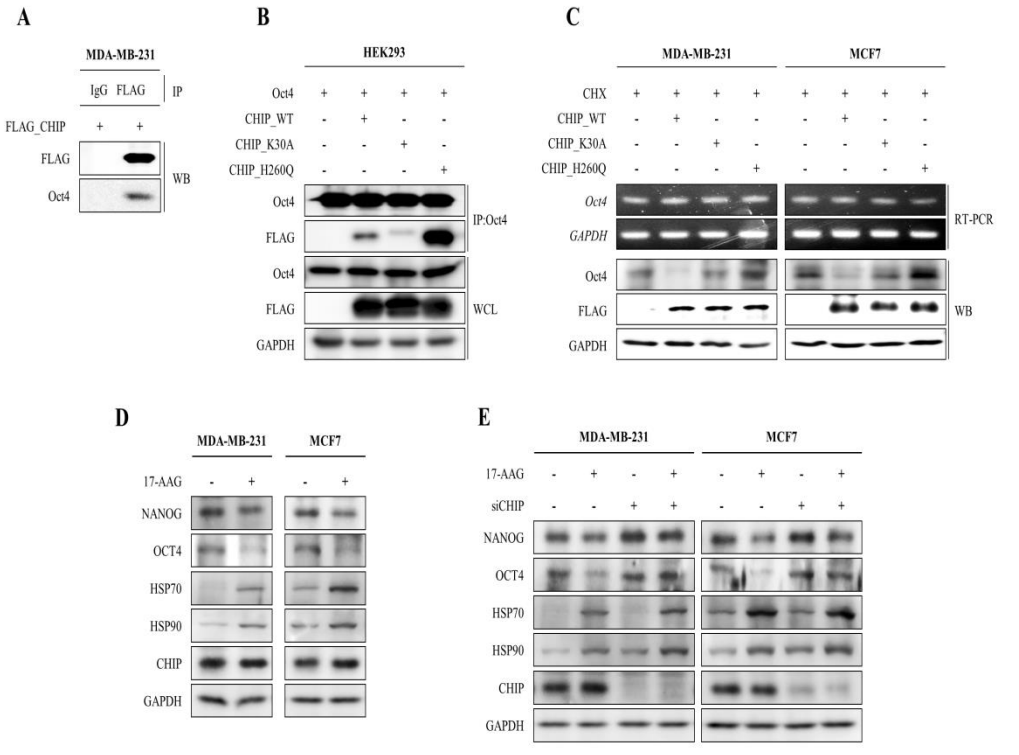



**Figure 3. The E3 ligase, CHIP interacts with OCT4.** (A) In the FLAG_CHIP transfected MDA-MB-231 cells, interaction between CHIP and OCT4 was detected by immunoprecipitation assay. (B) The HEK293 cells were transfected with FLAG_CHIP WT or indicated mutant constructs, K30A and H260Q, and co-transfected with *Oct4* expression vector. 48 hr after transfection, the interaction of OCT4 and CHIP (WT or mutant) was detected by immunoprecipitation assay. (C) The cells were transfected with indicated constructs. After 40 hr, transfected cells were treated with 20 μM CHX for an additional 8 hr. (D) The cells were treated with 5 μM 17-AAG for 8 hr. (E) The cells were transfected with scramble or CHIP siRNA. After 40 hr, transfected cells treated with 5 μM 17-AAG for an additional 8 hr.

Protein levels of indicated genes were detected by western blot. GAPDH was used as a loading control. The protein levels were detected by western blot.

### 4. CHIP E3 ligase induces poly-ubiquitination of OCT4

As the OCT4 levels are decreased in CHIP overexpressed cells, therefore, I performed ubiquitination assay in denaturing condition. In the CHIP depleted cells, the poly-ubiquitinated OCT4 was decreased (Fig. 4A and B), however, the increase of poly-ubiquitinated OCT4 was detected in the CHIP overexpressed cells (Fig. 4C and D). In addition, the poly-ubiquitinated OCT4 was not increased in CHIP mutant constructs, K30A and H260Q, overexpressed cells (Fig. 4E and F).

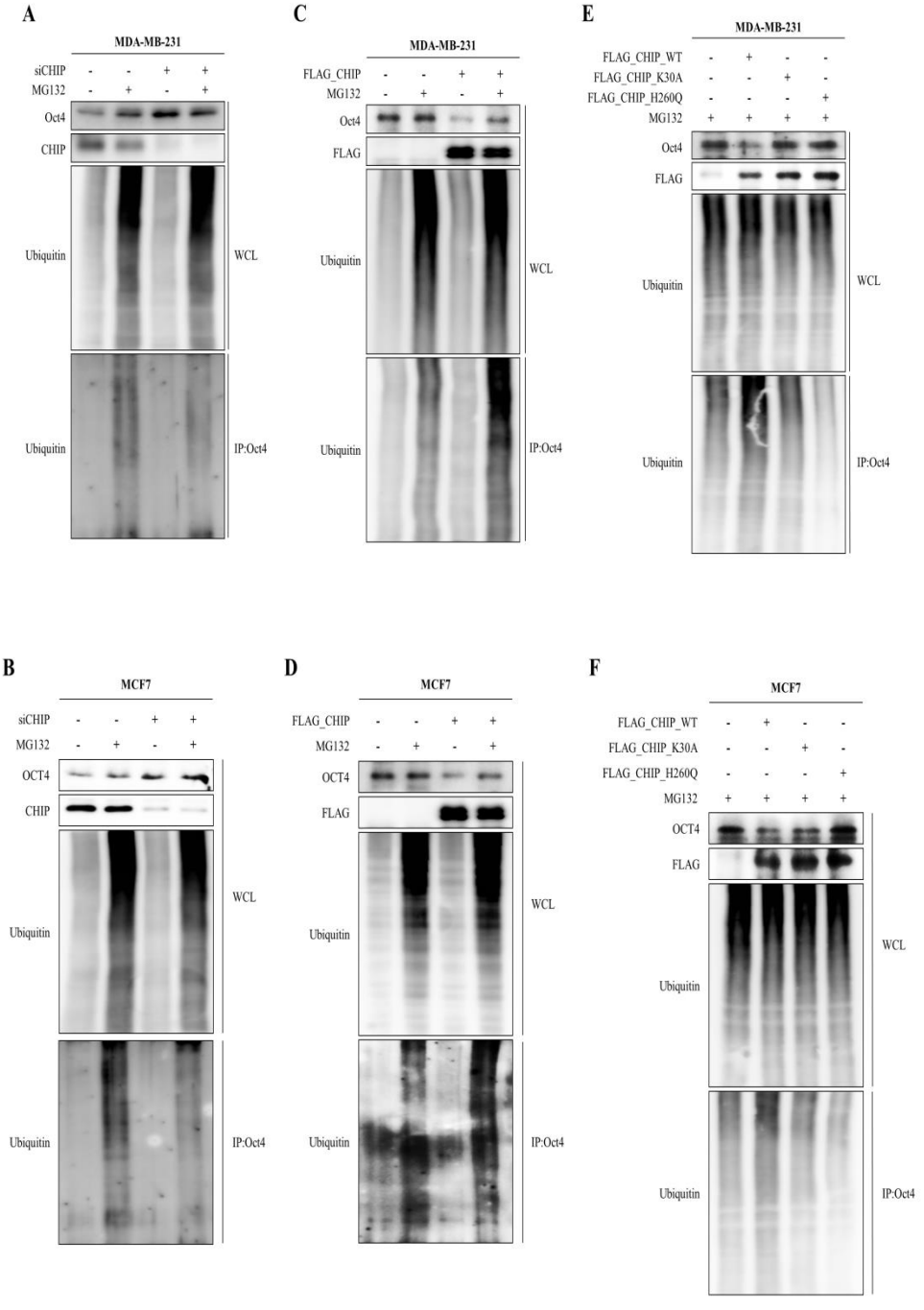


**Figure 4. The E3 ligase, CHIP interacts with OCT4 and induces poly-ubiquitination of OCT4.** The cells were transfected with indicated siRNA or vectors, CHIP siRNA (A and B), FLAG_CHIP overexpressing vector (C and D), and FLAG_CHIP WT or mutant overexpressing vectors (E and F). After 40 hr, transfected cells were treated with 20 μM MG132 for an additional 8 hr. The cell lysates were prepared in denaturing condition and were immunoprecipitated using OCT4 antibody. Poly-ubiquitination of OCT4 was detected by western blot.

## 5. 284 Lysine of OCT4 is an ubiquitination site by CHIP E3 ligase

Next, I explored the prediction of OCT4 ubiquitination site using the online resource (Table 3), and I identified ubiquitination on lysine 284 of OCT4 with diglycine modification in MS/MS spectrum (Fig. 5A). Thus, I generated the ubiquitination defective mutant construct on K284R. In the $Oct4_{KD}$ cells, the interaction between CHIP with OCT4_K284 was also detected (Fig. 5B), however, decrease of OCT4_K284R was not detected with CHX treatment and the increasing amount CHIP (Fig. 5C). In addition, the poly-ubiquitinated OCT4_K284R was not increased by CHIP overexpression, compared to OCT4 WT (Fig. 5D). These data indicate that the CHIP E3 ligase poly-ubiquitinates the OCT4 at K284.

**Table 3. The OCT4 is polyubiquitinated at K284 by CHIP that regulates the OCT4 stability**

| Peptide | Position | Score | Threshold |
|---|---|---|---|
| TVTPGAVKLEKEKLE | 123 | 2.53 | -1.9 |
| PGAVKLEKEKLEQNP | 126 | 0.91 | -1.9 |
| AVKLEKEKLEQNPEE | 128 | 1.17 | -1.9 |
| PEESQDIKALQKELE | 140 | 0.64 | -1.9 |
| QDIKALQKELEQFAK | 144 | 0.85 | -1.9 |
| KELEQFAKLLKQKRI | 151 | 0.64 | -1.9 |
| EQFAKLLKQKRITLG | 154 | 1.34 | -1.9 |
| FAKLLKQKRITLGYT | 156 | 1.35 | -1.9 |
| TLGVLFGKVFSQTTI | 177 | 1.23 | -1.9 |
| EALQLSFKNMCKLRP | 195 | 1.08 | -1.9 |
| LSFKNMCKLRPLLQK | 199 | 0.6 | -1.9 |
| KLRPLLQKWVEEADN | 206 | 0.25 | -1.9 |
| ENLQEICKAETLVQA | 222 | -0.78 | -1.9 |
| ETLVQARKRKRTSIE | 231 | 1.4 | -1.9 |
| LVQARKRKRTSIENR | 233 | 1.76 | -1.9 |
| NLFLQCPKPTLQQIS | 254 | 0.25 | -1.9 |
| AQQLGLEKDVVRVWF | 271 | -1.23 | -1.9 |
| **WFCNRRQKGKRSSSD** | **284** | **1.97** | **-1.9** |
| CNRRQKGKRSSSDYA | 286 | 1.77 | -1.9 |

Prediction of OCT4 ubiquitination sites using the online resource,

http://bdmpub.biocuckoo.org/prediction.php

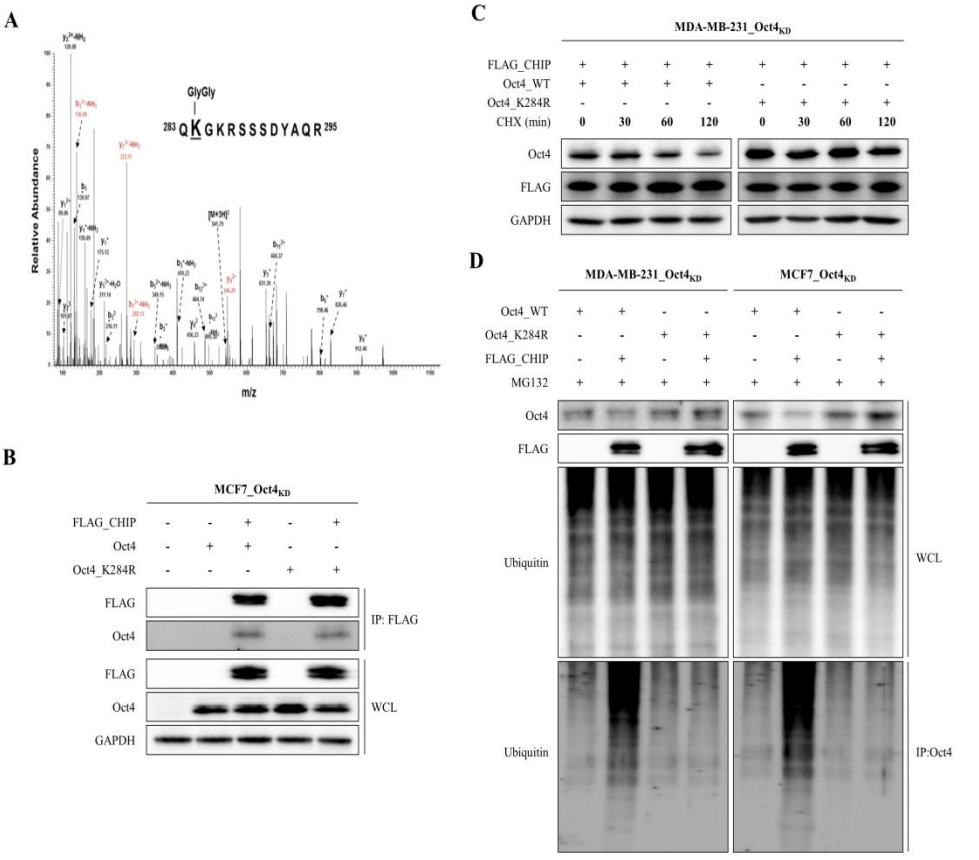



**Figure 5. The OCT4 is polyubiquitinated at K284 by CHIP that regulates the OCT4 stability.** (A) identified ubiquitination on lysine 284 of OCT4 with diglycine modification in MS/MS spectrum. (B) The MCF7_*Oct4*$_{KD}$ cells were transfected with indicated mutant constructs. 48 hr after transfection, the interaction of OCT4 (WT or mutant) and CHIP was detected by immunoprecipitation assay. (C) The cells were transfected with increasing amount FLAG_CHIP, and co-transfected with Oct4_WT or K284R. After 40 hr, transfected cells were treated with 20 μM CHX for an additional 8 hr. The protein levels of WT and mutated OCT4 and FLAG_CHIP were

detected by western blot. GAPDH was used as a loading control. (D) The cells were transfected with indicated constructs. After 40 hr, transfected cells were treated with 20 μM MG132 for an additional 8 hr. The cell lysates were prepared in denaturing condition and were immunoprecipitated using OCT4 antibody. Poly-ubiquitination of OCT4 was detected by western blot.

### 6. Overexpression of CHIP E3 ligase reduces cancer stem cell population

I measured the transcriptional activity of the *Oct4* promoter to check whether CHIP regulates the transcriptional activity of OCT4. The activity was enhanced by CHIP depletion, and repressed by CHIP overexpression (Fig. 6A). I also observed that the cell viability of each cell lines was increased by CHIP depletion, and reduced by additional OCT4 depletion, like as OCT4 depletion cells (Fig. 6B). Next, I confirmed the side population that is one of stemness feature, because the OCT4 is a potential CSC marker.[5] The side population was dramatically increased in CHIP depleted cells, however, dramatically decreased in OCT4 depleted cells (Fig. 6C). Interestingly, the side population was decreased in the both depletion of CHIP and OCT4 compared to CHIP depleted cells, whereas that was increased which compared to OCT4 depleted cells.

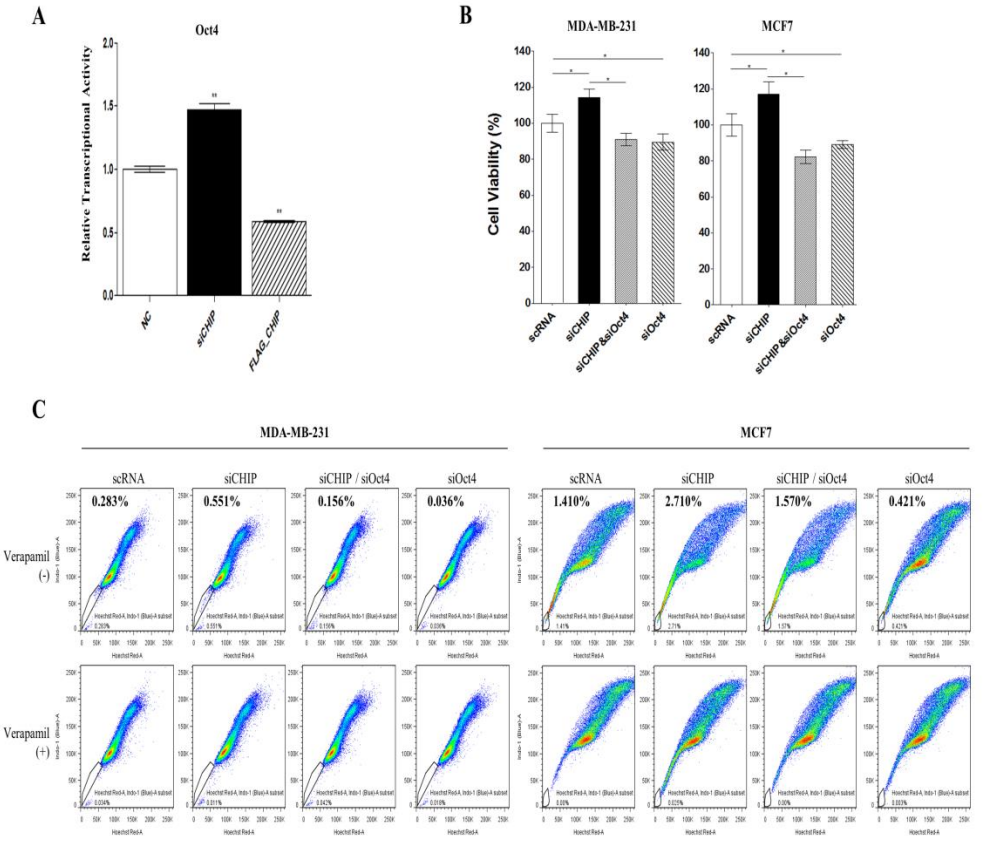



**Figure 6. The poly-ubiquitination at K284 of OCT4 by CHIP that regulates the cell viability and side population.** (A) Transcriptional activation was measured by reporter assay in MCF7 cells. Cells were transfected with *Oct4* reporter vector alone or co-transfected with CHIP siRNA or FLAG_CHIP expressing vector for 48 hr. (B) The cells were transfected with CHIP and Oct4 siRNA, alone or combination. 48 hr after transfection, the cell viability was detected by WST assay. (C) Side population analysis was performed in indicated siRNA transfected cells as described in 'materials and methods'. Data are presented as mean ± SD ($n$ = 3). Significant differences are

indicated by an asterisk (* $p<0.05$, ** $p<0.01$), and $p$ values were calculated using the Student's $t$ test.

### 7. Overexpression of ubiquitination mutant OCT4 increases cancer stem cell population

For the functional study of OCT4_K284R, I generated the depleted endogenous *Oct4* stable lines, $Oct4_{KD}$. I measured the cell viability of the $Oct4_{KD}$ cells (Fig. 7A). The viability was increased by OCT4 overexpression, and more increased by OCT4_K284R overexpression than OCT4 WT. Interestingly, the increased viability of OCT4 WT overexpressed cells was reduced by additional CHIP overexpression, however, that of OCT4_K284R was not. Next, I measured the side population whether different stabilities of OCT4 WT and K284R mutant influence the stemness of breast cancer cells. The side population was increased by OCT4 overexpression, and more increased by OCT4_K284R overexpression than OCT4 WT. Interestingly, the increased side population of OCT4 WT overexpressed cells was reduced by additional CHIP overexpression, however, that of OCT4_K284R was less reduced than OCT4 WT (Fig. 7B). These data indicate that the CHIP E3 ligase poly-ubiquitinates the OCT4 at K284, which influence the function of OCT4, known as a stemness factor.

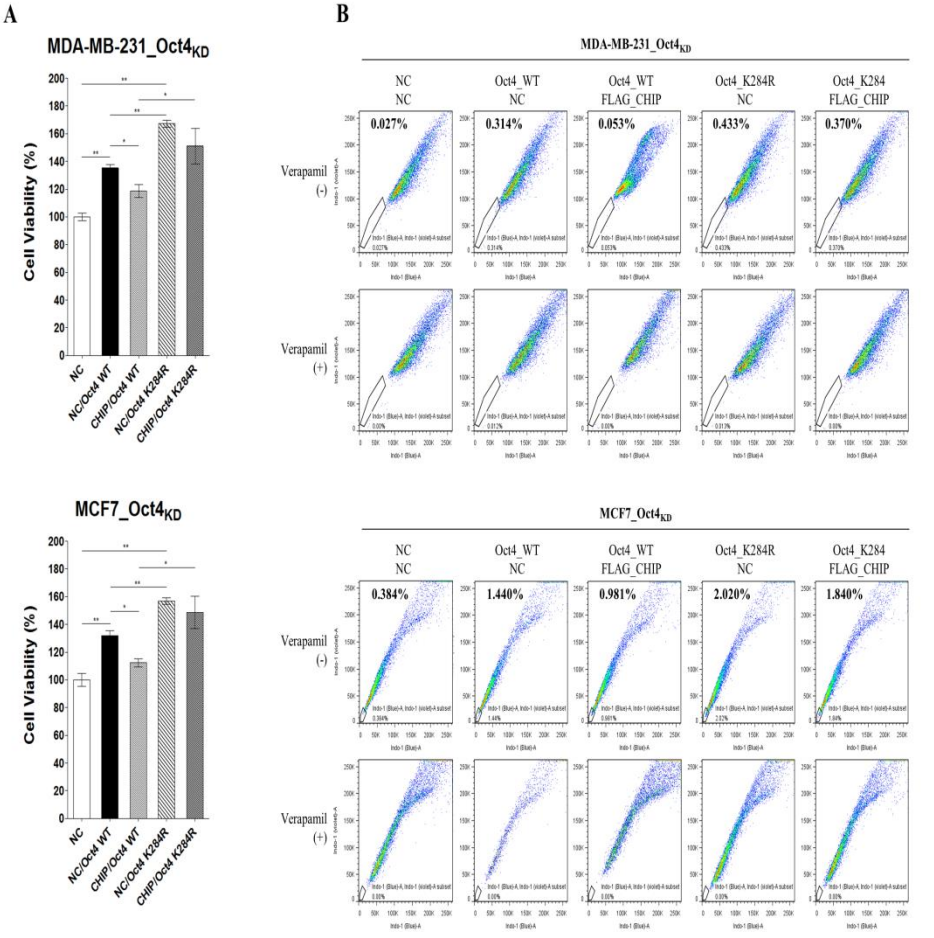


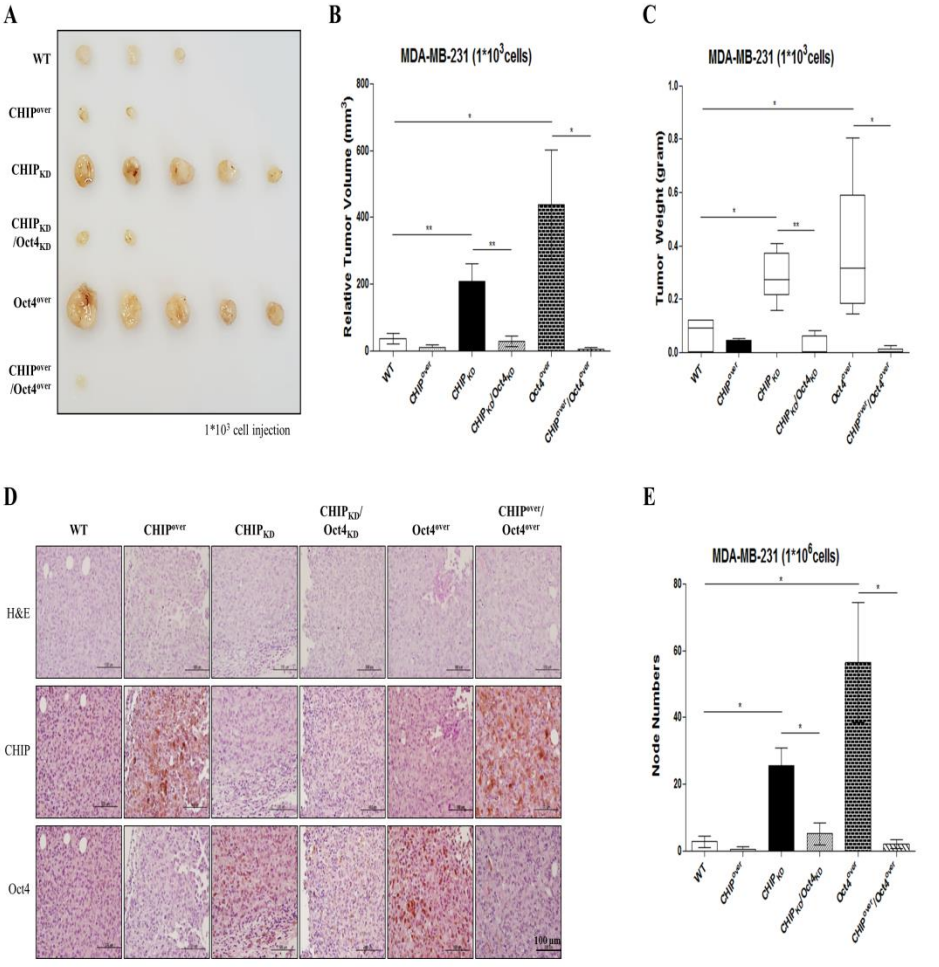

**Figure 7. The poly-ubiquitination at K284 of Oct4 by CHIP that regulates the cell viability and side population.** (A) The cells were transfected with indicated expression vectors, alone or combination. 48 hr after transfection, the cell viability was detected by WST assay. (B) The cells were transfected with indicated expression vectors, alone or combination. 48 hr after transfection, the side population analysis was performed as described in 'materials and methods'. Data are presented as mean ±

SD ($n$ = 3). Significant differences are indicated by an asterisk (* $p$<0.05, ** $p$<0.01), and $p$ values were calculated using the Student's $t$ test.

**8. Increased tumor burdens and metastasis by OCT4 overexpression are rescued by CHIP E3 ligase in breast cancer xenografted mice model**

To test the in vivo effect of CHIP on breast cancer tumorigenesis, I generated stable cell lines using MDA-MB-231 cells, and xenografted using mammosphere cultured with this cell line, 1 x $10^3$ cells. Whereas the xenografted tumor of CHIP overexpressed cells was not or detected smaller than WT cells, the largest tumor burdens were detected in CHIP depleted cells. The OCT4 overexpressed cell also promoted tumor growth in xenografted mice like as CHIP depleted cells or more than (Fig. 8A, B and C). However, the combined depletion or overexpression of CHIP and OCT4 cells derived tumors were not or detected small as well as CHIP overexpressed tumor. The reversely correlated expression of CHIP with OCT4 was detected in xenografted tumors by immunohistochemistry (Fig. 8D). Furthermore, additional xenografted tumors using different cell numbers showed similar with using 1 x $10^3$ cells (Table 4).

I also injected the indicated cells into the tail veins of mice, and counted colonies of tumor cells in their lungs (Fig. 8E). Whereas the metastatic tumors of WT and CHIP overexpressed cells were rarely detected, metastatic tumors derived from CHIP depletion and OCT4 overexpression cells were frequently detected in mice lungs (Fig. 8F and G). However, metastatic tumors derived from the combined depletion or overexpression of CHIP and OCT4 cells were rarely detected. These data show that the CHIP strongly decelerates tumor progression and metastasis *in vivo*, a similar finding to *in vitro*.



F


G

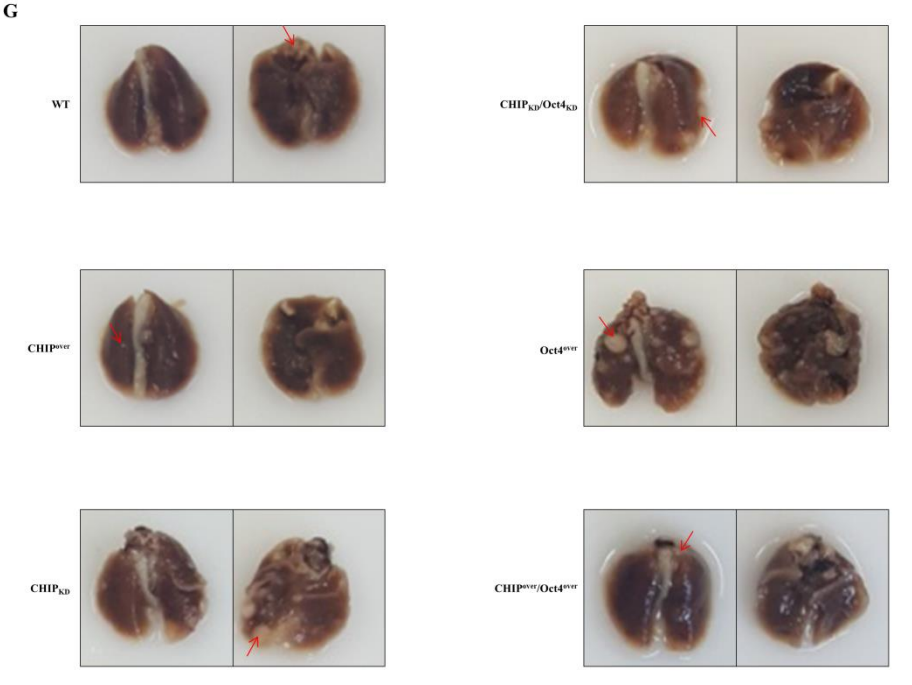


**Figure 8. OCT4 overexpressed cells derived tumor and metastatic node are reduced by CHIP overexpression *in vivo*.** Indicated stable lines of MDA-MB-231 cells (1 x $10^3$) were implanted into nude mice to form subcutaneous xenografts. After 12 weeks, tumors were isolated and presented in a photograph (A), measured the size (B) and weight (C) in a statistical graph. (D) Immunohistochemical analysis of xenografted tumors using CHIP and OCT4 antibodies. Scale bars, 100 μm. (E) Indicated stable lines of MDA-MB-231 cells (1 x $10^6$) were injected into tail vein of nude mice. After 12 weeks, lungs were isolated and counted the metastatic node numbers that are presented in a statistical graph, and presented in a photograph (F). (G) The expanded photograph of (F). Red arrows indicate the metastatic nodes. Data are presented as mean ± SD ($n$ = 5). Significant differences are indicated by an asterisk (* $p$<0.05, ** $p$<0.01), and $p$ values were calculated using the Student's $t$ test.

**Table 4. OCT4 overexpressed cells derived tumor and metastatic node are reduced by CHIP overexpression *in vivo***

| MDA-MB-231 | Tumorigenesis | | | Metastasis |
|---|---|---|---|---|
| | $10^3$ Cells | $10^4$ Cells | $10^5$ Cells | $10^6$ Cells |
| Wild Type | 3/5 | 4/5 | 5/5 | 3/5 |
| CHIPover | 2/5 | 3/5 | 4/5 | 1/5 |
| CHIPKD | 5/5 | 5/5 | 5/5 | 5/5 |
| CHIPKD/Oct4KD | 2/5 | 4/5 | 4/5 | 2/5 |
| Oct4over | 5/5 | 5/5 | 5/5 | 5/5 |
| CHIPover/Oct4over | 1/5 | 4/5 | 4/5 | 2/5 |

Indicated stable lines and numbers of MDA-MB-231 cells were implanted into nude mice to form subcutaneous xenografts or injected into the tail veins. After 12 weeks, the tumors and lungs were isolated and the number of mice with tumor or node bearing lungs was counted ($n$ = 5).

### 9. CHIP E3 ligase low-expressed breast cancer patients have poor survival probability

Finally, I explored the survival probability of breast cancer patients with *CHIP* expression using the online resource, Kaplan-Meier plot analysis. The *CHIP* low-expressed breast cancer patients showed the poor survival probability, such as overall, relapse free, distant metastasis free and post progression survival (Fig. 9). Furthermore, *Oct4* high-expressed breast cancer patients showed the poor post progression survival probability (Fig. 2C). These data support that the CHIP correlates with tumor progression and metastasis via OCT4 regulation.

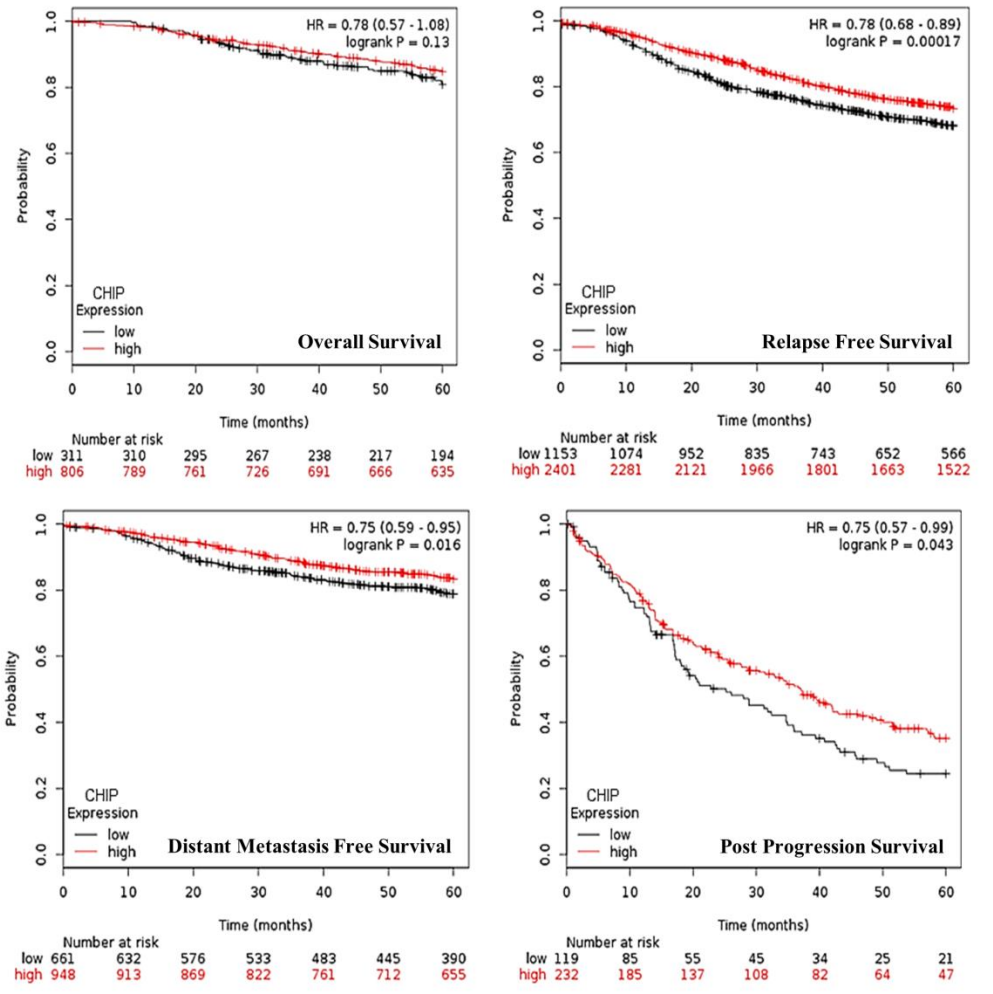



**Figure 9. Survival probability of breast cancer patients with CHIP expression.** Overall, relapse free, distant metastasis and post progression survival were analyzed with *CHIP* expression level using Kaplan-Meier plot analysis.

# IV. DISCUSSION

In this study, I established mammospheres from MDA-MB-231 and MCF7 breast cancer cells to characterize CSCs. Because CSCs likely are a minority cell population among total cancer cells, their identification and enrichment are a significant challenge.[13] This indicates that stem cells from breast tissue can be performed by the simple mammosphere cultures that enrich highly tumorigenic CSCs, which are probably the most dangerous cells within tumors compared with the bulk population of tumor cells, by using a simple *in vitro* culture technique. I observed that CHIP E3 ubiquitin ligase was significantly downregulated in mammospheres derived from MDA-MB-231 and MCF7 cells compared with that in monolayer cultured cells. I found that CHIP directly interacted with OCT4 and decreased its stability and breast CSC properties. OCT4, a transcription factor encoded by *POU5F1*, belongs to POU family of DNA-binding proteins.[35] This protein regulates target gene expression by binding to octamer motif ATGCAAAT within promoters or enhancers.[36] OCT4, whose expression is associated with pluripotent properties of stem cells, is an essential factor that controls the early stages of mammalian embryogenesis.[37] *Oct4* mRNA expression level in mouse embryonic stem cells is critical for the maintenance of pluripotency and differentiation toward trophoblast or primitive endodermal and mesodermal lineages.[38] Post-translational modification of OCT4 is important to regulate its function and to cure diseases.[39] Protein kinase A and/or MAPK phosphorylate OCT4 at highly conserved Ser229 (murine) or Ser236 (human) within

the POU DNA-binding homeodomain.[11] Phosphorylation at this serine residue sterically hinders both DNA binding and homodimer assembly. ERK1/2 phosphorylates OCT4 at Ser111 to regulate its subcellular distribution and degradation.[40] Small ubiquitin-related modifier (SUMO)-1 targets OCT4 at Lys118 in mice.[41] Sumoylation of OCT4, which does not alter its subnuclear localization, enhances its stability, DNA binding, and transactivation functions. E3 ligases interact directly with OCT4 to promote ubiquitin transfer. OCT4 ubiquitination promotes its degradation and dramatically regulates its transcriptional activity.[42-44] WWP2, Itch, and DPF2 are E3 ubiquitin ligases that specifically interact with OCT4. However, OCT4 site ubiquitinated by these ligases is unclear. The present study is the first to show that CHIP ubiquitinates OCT4 at Lys284. CHIP overexpression did not degrade OCT4_K284R. OCT4_K284R overexpression increased the proliferation and side population of breast cancer cells. These data strongly indicate that CHIP expression decreases in CSCs and that OCT4 dosage is critical for CSC maintenance. However, E3 ligases WWP2 and Itch may not maintain OCT4 stability in breast CSCs because expression of these E3 ligases is upregulated like that of OCT4 in mammosphere cultures. We believe that different E3 ligases regulate OCT4 stability differently in ES cells and CSCs. Therefore, we hypothesize that differential regulation of E3 ligases is critical for CSC survival and maintenance in tumors and needs to be examined further.

Regulation of CHIP in CSCs is unclear. Although I did not elucidate this in the present study, I have to discuss it. Limited evidence is available on the regulation of *CHIP* mRNA expression and post-translational modification under different

physiological and pathological contexts. *CHIP* mRNA expression is upregulated under various stress conditions such as heat shock and oxidative damage.[45,46] Stress-induced transcriptional regulation is important under various physiological conditions such as neurodegenerative disorders and heart diseases. TLR2 activation enhances CHIP expression and activity through JNK signaling.[47] Downregulation of *CHIP* mRNA and protein expression has been reported in malignant tissues compared with those in their normal counterparts, including the stomach,[48] pancreas,[49] and breast.[16] In MC3T3-E1 cells, miR-764-5p inhibits *CHIP* mRNA translation by binding at its 3′-UTR.[50] CHIP is downregulated in osteoblast progenitor cells during osteoblast differentiation. CHIP activity and stability are also regulated through post-translational modification. The N- and C-terminal regions of CHIP are proposed to contain functional phosphorylation sites, which should be confirmed in future studies. Association between CHIP and kinases such as ERK5 and Lim kinase 1 has been reported.[51,52] ERK5 activation increases CHIP ubiquitin ligase activity possibly through a conformational change in CHIP. Phosphorylation-dependent regulation of CHIP activity is an interesting possibility that needs further investigation. Moreover, mechanisms underlying decreased CHIP expression in CSCs and association between low CHIP expression and CSC survival should be investigated. At present, I am examining the regulation of CHIP expression and activation in CSCs.

Thus, these results indicate that CSCs show decreased CHIP expression and increased OCT4 stability through post-translational modifications to maintain their population and survival during breast cancer progression. These results also suggest

that targeting OCT4 post-translational modification is an ideal approach for breast cancer therapy.

# V.CONCLUSION

CHIP is known to function as a chaperone associated E3 ligase. Recently, CHIP is disputed regarding the oncogenic or tumor suppressive role. In the breast cancer, CHIP was reported whose expression was negatively correlated with tumor growth and metastasis.

This study hypothesizes that the CHIP could be one of the regulators in the cancer stemness, and demonstrates that the CHIP is regulator of OCT4 stability. In the mammosphere, one of stemness features, CHIP is downregulated, and influenced mammosphere forming ability. Notably, the OCT4 is identified in CHIP interactomes, and the CHIP regulates the OCT4 protein levels through the proteosomal degradation, independent of its mRNA level. The CHIP induces the OCT4 poly-ubiquitination at the K284. However, the poly-ubiquitination of K284R mutant OCT4 is not increased by the CHIP. In the breast cancer cells, the overexpression of CHIP reduces its cell proliferation and side population, however, K284R mutant OCT4 expressed cells are not reduced by CHIP overexpression. Moreover, whereas the xenografted tumor of CHIP overexpressed cells is not or detected smaller than WT cells, the largest tumor burdens is detected in CHIP depleted cells. The combined depletion or overexpression of CHIP and OCT4 cells derived tumors are not detected or detected small as well as CHIP overexpressed tumor. Finally, in the online resource, Kaplan-Meier plot analysis, The *CHIP* low-expressed breast cancer patients showed the poor survival probability, post progression survival.

Taken all together, ubiquitination of OCT4 by CHIP plays a role in breast tumorigenesis and I propose that the regulation of OCT4 stability could be a potent approach in breast cancer therapy.

# REFERENCES

1. Tang Y, Wang Y, Kiani MF, Wang B. Classification, Treatment Strategy, and Associated Drug Resistance in Breast Cancer. Clin Breast Cancer 2016; doi:10.1016/j.clbc.2016.05.012.
2. Marquette C, Nabell L. Chemotherapy-resistant metastatic breast cancer. Curr Treat Options Oncol 2012;13:263-75.
3. Carnero A, Garcia-Mayea Y, Mir C, Lorente J, Rubio IT, ME LL. The cancer stem-cell signaling network and resistance to therapy. Cancer Treat Rev 2016;49:25-36.
4. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, et al. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. Nat Rev Clin Oncol 2015;12:445-64.
5. Yang F, Xu J, Tang L, Guan X. Breast cancer stem cell: the roles and therapeutic implications. Cell Mol Life Sci 2016; doi:10.1007/s00018-016-2334-7.
6. Scholer HR, Dressler GR, Balling R, Rohdewohld H, Gruss P. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. EMBO J 1990;9:2185-95.
7. Hansis C, Grifo JA, Krey LC. Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. Mol Hum Reprod 2000;6:999-1004.

8. Li SW, Wu XL, Dong CL, Xie XY, Wu JF, Zhang X. The differential expression of OCT4 isoforms in cervical carcinoma. PLoS One 2015;10:e0118033.

9. Ng HH, Surani MA. The transcriptional and signalling networks of pluripotency. Nat Cell Biol 2011;13:490-6.

10. Saxe JP, Tomilin A, Scholer HR, Plath K, Huang J. Post-translational regulation of Oct4 transcriptional activity. PLoS One 2009;4:e4467.

11. Brumbaugh J, Hou Z, Russell JD, Howden SE, Yu P, Ledvina AR, et al. Phosphorylation regulates human OCT4. Proc Natl Acad Sci U S A 2012;109:7162-8.

12. Gilbertson RJ, Graham TA. Cancer: Resolving the stem-cell debate. Nature 2012;488:462-3.

13. Saadin K, White IM. Breast cancer stem cell enrichment and isolation by mammosphere culture and its potential diagnostic applications. Expert Rev Mol Diagn 2013;13:49-60.

14. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253-70.

15. Paul I, Ghosh MK. A CHIPotle in physiology and disease. Int J Biochem Cell Biol 2015;58:37-52.

16. Kajiro M, Hirota R, Nakajima Y, Kawanowa K, So-ma K, Ito I, et al. The ubiquitin ligase CHIP acts as an upstream regulator of oncogenic pathways. Nat Cell Biol 2009;11:312-9.

17. Jang KW, Lee KH, Kim SH, Jin T, Choi EY, Jeon HJ, et al. Ubiquitin ligase CHIP induces TRAF2 proteasomal degradation and NF-kappaB inactivation to regulate breast cancer cell invasion. J Cell Biochem 2011;112:3612-20.

18. Kang SA, Cho HS, Yoon JB, Chung IK, Lee ST. Hsp90 rescues PTK6 from proteasomal degradation in breast cancer cells. Biochem J 2012;447:313-20.

19. Schulz R, Marchenko ND, Holembowski L, Fingerle-Rowson G, Pesic M, Zender L, et al. Inhibiting the HSP90 chaperone destabilizes macrophage migration inhibitory factor and thereby inhibits breast tumor progression. J Exp Med 2012;209:275-89.

20. Su CH, Lan KH, Li CP, Chao Y, Lin HC, Lee SD, et al. Phosphorylation accelerates geldanamycin-induced Akt degradation. Arch Biochem Biophys 2013;536:6-11.

21. Paul I, Ahmed SF, Bhowmik A, Deb S, Ghosh MK. The ubiquitin ligase CHIP regulates c-Myc stability and transcriptional activity. Oncogene 2013;32:1284-95.

22. Bento CF, Fernandes R, Ramalho J, Marques C, Shang F, Taylor A, et al. The chaperone-dependent ubiquitin ligase CHIP targets HIF-1alpha for degradation in the presence of methylglyoxal. PLoS One 2010;5:e15062.

23. Li F, Xie P, Fan Y, Zhang H, Zheng L, Gu D, et al. C terminus of Hsc70-interacting protein promotes smooth muscle cell proliferation and survival through ubiquitin-mediated degradation of FoxO1. J Biol Chem 2009;284:20090-8.

24. Ahmed SF, Deb S, Paul I, Chatterjee A, Mandal T, Chatterjee U, et al. The chaperone-assisted E3 ligase C terminus of Hsc70-interacting protein (CHIP) targets PTEN for proteasomal degradation. J Biol Chem 2012;287:15996-6006.

25. Seo J, Lee EW, Sung H, Seong D, Dondelinger Y, Shin J, et al. CHIP controls necroptosis through ubiquitylation- and lysosome-dependent degradation of RIPK3. Nat Cell Biol 2016;18:291-302.

26. Cho Y, Lee HW, Kang HG, Kim HY, Kim SJ, Chun KH. Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer. Oncotarget 2015;6:8709-21.

27. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-72.

28. Lee EW, Lee MS, Camus S, Ghim J, Yang MR, Oh W, et al. Differential regulation of p53 and p21 by MKRN1 E3 ligase controls cell cycle arrest and apoptosis. EMBO J 2009;28:2100-13.

29. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 2006;1:2856-60.

30. Lee HW, Jang KS, Choi HJ, Jo A, Cheong JH, Chun KH. Celastrol inhibits gastric cancer growth by induction of apoptosis and autophagy. BMB Rep 2014;47:697-702.

31. Wang YG, Kim SJ, Baek JH, Lee HW, Jeong SY, Chun KH. Galectin-3 increases the motility of mouse melanoma cells by regulating matrix metalloproteinase-1 expression. Exp Mol Med 2012;44:387-93.

32. Takeshi Kotake YK, Sachiko Takahara, Shigeru Tsuyuki, Hiroshi Yoshibayashi, Eiji Suzuki, Yoshio Moriguchi, Hiroyasu Yamashiro, Kazuhiko Yamagami, Hirofumi Suwa, Toshitaka Okuno, Takahito Okamura, Takashi Hashimoto, Hironori Kato, Akihito Tsuji, Masakazu Toi. Impact of Eribulin Monotherapy on Post-Progression Survival in Patients with HER2-Negative Advanced or Metastatic Breast Cancer. International Journal of Cancer and Clinical Research 2016;3:1-5.

33. Saad ED, Katz A, Buyse M. Overall Survival and Post-Progression Survival in Advanced Breast Cancer: A Review of Recent Randomized Clinical Trials. Journal of Clinical Oncology 2010;28:1958-62.

34. Kumar P, Pradhan K, Karunya R, Ambasta RK, Querfurth HW. Cross-functional E3 ligases Parkin and C-terminus Hsp70-interacting protein in neurodegenerative disorders. J Neurochem 2012;120:350-70.

35. Zeineddine D, Hammoud AA, Mortada M, Boeuf H. The Oct4 protein: more than a magic stemness marker. Am J Stem Cells 2014;3:74-82.

36. Zhao FQ. Octamer-binding transcription factors: genomics and functions. Front Biosci (Landmark Ed) 2013;18:1051-71.

37. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998;95:379-91.

38. Shi G, Jin Y. Role of Oct4 in maintaining and regaining stem cell pluripotency. Stem Cell Res Ther 2010;1:39.

39. Wang YC, Peterson SE, Loring JF. Protein post-translational modifications and regulation of pluripotency in human stem cells. Cell Res 2014;24:143-60.

40. Spelat R, Ferro F, Curcio F. Serine 111 phosphorylation regulates OCT4A protein subcellular distribution and degradation. J Biol Chem 2012;287:38279-88.

41. Wei F, Scholer HR, Atchison ML. Sumoylation of Oct4 enhances its stability, DNA binding, and transactivation. J Biol Chem 2007;282:21551-60.

42. Liao B, Jin Y. Wwp2 mediates Oct4 ubiquitination and its own auto-ubiquitination in a dosage-dependent manner. Cell Res 2010;20:332-44.

43. Liao B, Zhong X, Xu H, Xiao F, Fang Z, Gu J, et al. Itch, an E3 ligase of Oct4, is required for embryonic stem cell self-renewal and pluripotency induction. J Cell Physiol 2013;228:1443-51.

44. Liu C, Zhang D, Shen Y, Tao X, Liu L, Zhong Y, et al. DPF2 regulates OCT4 protein level and nuclear distribution. Biochim Biophys Acta 2015;1853:3279-93.

45. Dikshit P, Jana NR. The co-chaperone CHIP is induced in various stresses and confers protection to cells. Biochem Biophys Res Commun 2007;357:761-5.

46. Stankowski JN, Zeiger SL, Cohen EL, DeFranco DB, Cai J, McLaughlin B. C-terminus of heat shock cognate 70 interacting protein increases following stroke and impairs survival against acute oxidative stress. Antioxid Redox Signal 2011;14:1787-801.

47. Meng Y, Chen C, Wang L, Wang X, Tian C, Du J, et al. Toll-like receptor-2 ligand peptidoglycan upregulates expression and ubiquitin ligase activity of CHIP through JNK pathway. Cell Physiol Biochem 2013;32:1097-105.

48. Liu F, Zhou J, Zhou P, Chen W, Guo F. The ubiquitin ligase CHIP inactivates NF-kappaB signaling and impairs the ability of migration and invasion in gastric cancer cells. Int J Oncol 2015;46:2096-106.

49. Wang T, Yang J, Xu J, Li J, Cao Z, Zhou L, et al. CHIP is a novel tumor suppressor in pancreatic cancer through targeting EGFR. Oncotarget 2014;5:1969-86.

50. Guo J, Ren F, Wang Y, Li S, Gao Z, Wang X, et al. miR-764-5p promotes osteoblast differentiation through inhibition of CHIP/STUB1 expression. J Bone Miner Res 2012;27:1607-18.

51. Woo CH, Le NT, Shishido T, Chang E, Lee H, Heo KS, et al. Novel role of C terminus of Hsc70-interacting protein (CHIP) ubiquitin ligase on inhibiting cardiac apoptosis and dysfunction via regulating ERK5-mediated degradation of inducible cAMP early repressor. Faseb j 2010;24:4917-28.

52. Lim MK, Kawamura T, Ohsawa Y, Ohtsubo M, Asakawa S, Takayanagi A, et al. Parkin interacts with LIM Kinase 1 and reduces its cofilin-phosphorylation activity via ubiquitination. Exp Cell Res 2007;313:2858-74.

# ABSTRACT (IN KOREAN)

## E3 ligase CHIP 에 의한 OCT4 의 ubiquitination 조절 기전과 유방암의 종양발생에 미치는 영향 연구

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유방암의 발생의 여러 가지 원인들 중, 하나로 꼽히는 종양줄기세포는 특히 재발하는 유방암에서도 주요한 원인으로 각광받고 있다. 따라서 종양줄기세포의 특징 및 조절에 대한 연구가 최근에 주목되고 있다. 이러한 기존의 연구들을 바탕으로 본 연구에서는 먼저 종양줄기세포의 특징을 나타내는 mammosphere 배양세포를 이용하여 DNA microarray 분석을 한 결과, CHIP 의 발현이 mammosphere 에서 감소하는 것을 확인하였다. 또한 CHIP 의 발현을 감소시킨 세포주에서는 mammosphere 의 형성이 감소하지만, CHIP 이 과 발현 된 세포주를

이용하였을 때에는 증가하는 것을 관찰하였다. CHIP 과 결합하는 단백질들을 mass-spectrum 분석을 통하여 확인하였을 때, OCT4 가 CHIP 과 결합한다는 것을 확인하였다. 또한 CHIP 에 의하여 OCT4 의 안정화가 저해되고, 이는 Oct4 의 poly-ubiquitination 및 프로테아좀 분해에 의한 것임을 확인하였다. 이러한 현상은 284 번째 lysine 에서 일어나는 것을 확인하였다. 이와 관련하여 WT OCT4 를 발현할 때는 CHIP 이 과 발현되면 세포 증식과 side population 이 감소하지만, K284R OCT4 가 발현할 때는 이러한 현상이 나타나지 않았다. 또한 xenograft 실험에서도 같은 현상을 관찰하였고, 유방암 환자들에서도 CHIP 의 발현이 낮을 경우 생존율이 좋지 않은 것을 확인하였다.

이러한 결과들은 CHIP 에 의한 OCT4 의 ubiquitination 이 유방암 발생에서 중요한 역할을 하는 것을 의미한다. 따라서 CHIP 에 의한 OCT4 단백질의 안정화 조절 기전은 유방암 치료에 있어서 새로운 표적으로 제시될 수 있다.

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핵심되는 말: CHIP, Ubiquitination, OCT4, 종양발생, 유방암

# PUBLICATION LIST

1. Kim SJ, Lee HW, Baek JH, **Cho YH**, Kang HG, Jeong JS, et al. Activation of nuclear PTEN by inhibition of Notch signaling induces G2/M cell cycle arrest in gastric cancer. Oncogene 2016;35:251-60.
2. Kim HY, **Cho Y**, Kang H, Yim YS, Kim SJ, Song J, et al. Targeting the WEE1 kinase as a molecular targeted therapy for gastric cancer. Oncotarget 2016;7:49902-16.
3. Kang HG, Kim DH, Kim SJ, **Cho Y**, Jung J, Jang W, et al. Galectin-3 supports stemness in ovarian cancer stem cells by activation of the Notch1 intracellular domain. Oncotarget 2016;7:68229-41.
4. **Cho Y**, Lee HW, Kang HG, Kim HY, Kim SJ, Chun KH. Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer. Oncotarget 2015;6:8709-21.