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Cellular inhibitor of apoptosis protein 2 promotes  
the epithelial-mesenchymal transition in triple-  
negative breast cancer cells through activation of  
the AKT signaling pathway

Su Ji Jo

Department of Medical Science  
The Graduate School, Yonsei University

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Directed by Professor Jae Myun Lee

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

Su Ji Jo

June 2017

This certifies that the Master's Thesis of  
Su Ji Jo is approved.

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Thesis Supervisor: Jae Myun Lee

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Thesis Committee Member #1: Joon Jeong

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Thesis Committee Member #2: Ja Seung Koo

The Graduate School  
Yonsei University

June 2017

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그리고 저에게 많은 도움을 주신 미생물학교실 선생님들께 감사의 말을 전하고 싶습니다. 실험의 기본도 모르는 저에게 실험을 알려준 혜미 언니, 이현규 선생님, 학위를 시작하고 방황하던 저에게 따뜻한 손길을 내밀어 준 조용근 선생님, 양창모 선생님께 감사의 말씀을 꼭 드리고 싶습니다. 본 연구를 이끌어 주시고 항상 격려해 주신 박필구 선생님께 정말 감사 드립니다. 박필구 선생님의 도움이 없었다면 저는 이 자리에 올 수 없었을 것입니다. 그리고 본 연구의 기초를 다져 주신 차혜란 선생님, 많은 조언을 해주신 김민정 선생님, 심두희 선생님, 문국영 선생님께 감사하다는 말씀을 드립니다. 그리고 같은 공간에서 함께 지내며 정든 설지희 선생님, 수진이에게 고마운 마음을 전합니다.

마지막으로 언제나 저를 믿어주고 사랑해 주시는 부모님과 동생들에게 이 논문을 바칩니다.

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## ABSTRACT

Cellular inhibitor of apoptosis protein 2 promotes the epithelial-mesenchymal transition in triple-negative breast cancer cells through activation of the AKT signaling pathway

Su Ji Jo

Department of Medical Science  
The Graduate School, Yonsei University

(Directed by Professor Jae Myun Lee)

Triple-negative breast cancer (TNBC) represents approximately 10–17% of all breast cancers, and patients with TNBC show a poorer short-term prognosis than patients with other types of breast cancer. TNBCs also have a higher tendency for early distant metastasis and cancer recurrence due to induction of the epithelial-mesenchymal transition (EMT). Several recent reports have suggested that inhibitor of apoptosis (IAP) proteins function as regulators of the EMT. However, the roles of these proteins in TNBC are not clear. Accordingly, I

investigated the roles of cIAP2 in TNBC. Among eight IAP genes, only cIAP2 was upregulated in TNBC cells compared with that in other breast cancer subtypes. Analysis of TMAs revealed that expression of cIAP2 was upregulated in TNBCs. In vitro studies showed that cIAP2 was highly expressed in TNBC cells compared with that in other types of breast cancer cells. Furthermore, silencing of cIAP2 in TNBC cells induced mesenchymal-epithelial transition (MET)-like processes and subsequently suppressed the migratory ability and invasion capacity of the cells by regulation of Snail through the AKT signaling pathway. In contrast, ectopic expression of cIAP2 in luminal-type breast cancer cells induced activation of the AKT signaling pathway. These results collectively indicated that cIAP2 regulated the EMT in TNBC via activation of the AKT signaling pathway, contributing to metastasis in TNBC. I propose a novel mechanism through which cIAP2 regulates the EMT involving AKT signaling in TNBC cells. I suggest that cIAP2 may be an attractive candidate molecule for the development of targeted therapeutics in the future.

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Keywords: Triple-negative breast cancer, cellular inhibitor of apoptosis protein 2, epithelial-mesenchymal transition, AKT signaling pathway

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## I. INTRODUCTION

Breast cancer is currently one of the most common cancers. Worldwide, approximately 22% of new cancers are diagnosed as breast cancer, and the number of patients with breast cancer has reached about 3.9 million. In women, breast cancer is a leading cause of mortality, with approximately 520,000 annual deaths.<sup>1</sup> With the recent success of therapy based on

molecular targeted drugs against breast cancer<sup>2</sup>, further improvements in the efficacy of anticancer treatments are expected. However, there are no cure-all cancer therapies. Many types of breast cancers do not respond to current treatments, making the effective treatment of cancer difficult.

Clinically, breast cancer is usually categorized based on the expression of specific receptors, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)/neu (also known as ERBB2). Cancers expressing none of these three receptors are referred to as triple-negative breast cancers (TNBCs), which account for 10–17% of all breast cancers.<sup>3</sup> TNBCs show a relatively poorer prognosis compared with other types of breast cancer and often cannot be subjected to current targeted therapies due to the absence of receptors, which can act as therapeutic target molecules. Additionally, TNBCs are known to be associated with a higher tendency of progression to metastatic diseases, the major cause of breast cancer-related death.<sup>4</sup> The lack of efficient targeted therapy and higher potential for metastatic progression of TNBCs need to be overcome in order to improve breast cancer therapies. To conquer breast cancer, novel strategies based on molecular mechanisms associated with TNBC will be necessary.

Cancer metastasis is a multicellular process involving a series of steps from primary cancer to metastatic malignancy at distal sites. During metastasis, cancer cells in primary tumors must change from the epithelial phenotype to the mesenchymal phenotype in order to penetrate into the basement membrane, a process called the epithelial-mesenchymal transition (EMT).<sup>5</sup> Many studies have shown that transcription factors, including Snail/Slug, Twist, and Zeb family proteins, govern the overall EMT process by regulation of various structural and adhesion/junction molecules.<sup>6</sup> Because the EMT is a key contributor to the metastatic process,

its regulatory mechanisms should be well organized and controlled. Although various transcription factors and their target genes have been extensively studied in the EMT processes, signaling molecules associated with the EMT are relatively poorly understood. Recently, several studies have reported novel molecules with EMT regulatory functions.<sup>7-9</sup>

Inhibitor of apoptosis proteins (IAPs), also known as baculovirus IAP repeat (BIR) domain-containing proteins (BIRCs), are a protein family including eight proteins commonly possessing BIR domains, including BIRC1 (NAIP), BIRC2 (cIAP1), BIRC3 (cIAP2), BIRC4 (XIAP), BIRC5 (survivin), BIRC6 (BRUCE), BIRC7 (livin), and BIRC8 (ILP2).<sup>10</sup> IAPs many function as E3 ligases owing to their C-terminal RING domains and are able to degrade proteins by linking them to ubiquitin molecules.<sup>11</sup> Because XIAP, cIAP1, and cIAP2 are directly or indirectly related to the apoptosis pathway through inhibition of caspases, many drugs for cancer therapy have focused on inhibition of IAPs.<sup>12</sup>

Recently, several reports have suggested the possibility of another function of IAPs as regulators of the EMT.<sup>13</sup> However, the function of IAPs related to the EMT process and cancer metastasis is still unclear. XIAP and cIAP2 are thought to be positive regulators of the EMT.<sup>14-16</sup> In contrast, one study showed that XIAP and cIAP1 inhibit the EMT by inducing Rac1 degradation.<sup>17</sup> Thus, because of the inconsistencies in clinical analysis of IAPs in cancer prognosis, further analyses are needed to determine the roles of IAPs in cancer progression and metastasis.

In this study, I evaluated the expression patterns of IAPs in several breast cancer cell lines. Subsequently, I examined the role of cIAP2 in TNBC and the EMT. These findings provide important insights into the mechanisms through which cIAP2 modulates the EMT in TNBC.

## II. MATERIALS AND METHODS

### 1. Cell culture

Human breast cancer cell lines, including MCF7, T-47D, BT474, SK-BR-3, Hs 578T, and MDA-MB-231 cells, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in accordance with ATCC recommendations. Cell culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 10 U/mL penicillin-streptomycin (Hyclone). The cells were cultivated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Characteristics of the breast cancer cell lines used in this study are described in Table 1.

### 2. Construction of cIAP2-siRNA and cIAP2 expression vector

For knockdown of cIAP2 (NM\_001165) mRNA, the following sequences were used: si-cIAP2, 5'-GGAGAGAAUUAUAGACUAGUCAATG-3'; si-Control (nonspecific oligo), 5'-CGUAAUCGCGUAUAAUACGCGUA-3' (IDT, Coralville, IA, USA). MDA-MB-231 and Hs 578T cells were transfected with cIAP2-siRNA or control-siRNA at 50% cell confluence using Lipofectamine RNAi MAX (Invitrogen, Green Island, NY, USA) according to the manufacturer's instructions. The optimal concentrations of siRNA for gene silencing were 50 nM for MDA-MB-231 cells and 25 nM for Hs 578T cells.

For ectopic expression of wild-type cIAP2, the pRK5-Flag-cIAP2 plasmid construct was purchased from Addgene (Cambridge, MA, USA). pcDNA 3.1-cIAP2 RING domain mutant (K558/559V) was purchased from ATGen (ATGen, Gyeonggi-do, Korea) and moved to the pRK5-Flag vector using xba I and HindIII enzyme. The pRK5-Flag vector was used as a

control. The plasmids were transfected into breast cancer cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

### 3. Cell proliferation assay

Cell proliferation rates were measured using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. Briefly, cIAP2-silenced TNBC cells were plated in 96-well microplates at a density of  $3 \times 10^3$  cells/well for MDA-MB-231 cells and  $1 \times 10^3$  cells/well for Hs 578T cells. Next, 10  $\mu$ L CCK-8 solution (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2, 4-disulfophenyl]-2H-tetrazolium) was added to each well. The plates were incubated at 37°C in an incubator for 3 hr, and the absorbance at 450 nm was measured using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

### 4. Western blot analysis

Cell lysates were prepared in RIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with protease inhibitors. The protein concentration was determined using a BCA assay kit (Thermo Fisher, Waltham, MA, USA). Whole-cell extracts were boiled in 4 $\times$  SDS sample buffer (2% SDS, 50 mM Tris-HCl [pH 6.8], 0.4 mM ethylenediaminetetraacetic acid [EDTA, pH 8.5], 10% glycerol, 0.002% pyronin Y) supplemented with 2-mercaptoethanol. The denatured protein was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to 0.45- $\mu$ m nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) by semidry transfer. The membranes were washed with Tris-buffered saline containing

0.05% Tween 20 (TBS-T) and blocked for 1 hr in TBS-T containing 5% skim milk. The membranes were then incubated with primary antibodies diluted with TBS-T at 4°C overnight and then washed three times with TBS-T. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in TBS-T containing 5% skim milk at room temperature for 90 min, followed by five washes with TBS-T. Finally, protein bands were visualized using WesternBright ECL Chemiluminescent HRP Substrate (Advansta, Menlo Park, CA, USA).

Primary antibodies were as follows: anti-cIAP2 (#3130; Cell Signaling Technology, Danvers, MA, USA), anti-E-cadherin (610181; BD Biosciences, Sparks, MD, USA), anti-epithelial cell adhesion molecule (EpCAM; ab71916; Abcam, Milton, Cambridge, UK), anti-N-cadherin (33-3900; Invitrogen), anti- $\alpha$ -smooth muscle actin (SMA; RB-9010-P; Invitrogen), anti-vimentin (M0725; Dako, Glostrup, Denmark), anti-Slug (#9585; Cell Signaling Technology), anti-Snail (#3895; Cell Signaling Technology), anti-phospho-AKT (Ser473; #4058; Cell Signaling Technology), anti-total AKT (#4691; Cell Signaling Technology), anti-phospho-glycogen synthase kinase (GSK) 3 $\beta$  (Ser9; #5558; Cell Signaling Technology), anti-GSK3 $\beta$  (#9315; Cell Signaling Technology), anti-phospho-inhibitor of kappa B alpha (I $\kappa$ B $\alpha$ ; Ser32; #2859; Cell Signaling Technology), anti-I $\kappa$ B $\alpha$  (#9242; Cell Signaling Technology), anti- $\beta$ -actin (AC-15; Sigma Aldrich, St. Louis, MO, USA).

## 5. Immunohistochemical (IHC) staining

TNBC tissue microarray (TMA) slides (100 cases) were obtained from Gangnam Severance Hospital (Yonsei University, Seoul, Korea). The Institutional Review Board of Gangnam Severance Hospital approved this study in accordance with good clinical practice

guidelines and the Declaration of Helsinki (3-2014-0239).

An OPTIVIEW Universal DAB kit (Ventana Medical Systems, Tucson, AZ, USA) was used for IHC staining. Slides were deparaffinized and rehydrated with sequential incubation in xylene, 100% to 70% ethanol, and distilled water. After antigen retrieval with cell conditioner 1 for 24 min at 100°C, slides were immersed with 3% H<sub>2</sub>O<sub>2</sub> for 4 min at 37°C and probed with anti-cIAP-2 antibodies (NB100-56132; Novus International, St. Charles, MO, USA) for 16 min at 37°C. OptiView HQ Universal Linker and OptiView HRP Multimer were sequentially added to the slides for 8 min each. Subsequently, 3,3'-diaminobenzidine (DAB) substrate solution was added. Slides were counterstained with hematoxylin and bluing reagent. Digital images of slides with DAB staining were obtained using Leica Application Suite (LAS) Microscope Software (Leica Microsystems Inc., Buffalo Grove, IL, USA).

IHC staining for cIAP2 was classified as negative (0), 1+, or 2+ in high-magnification (200×) fields according to the intensity of cytoplasmic staining and using quantitative scoring methods.<sup>18</sup> The scoring methods used in this study are described in Table 2. The cIAP2-positive status was assigned for scores 1+ and 2+. The interpretation of IHC staining was evaluated by a pathologist who had no information regarding the clinical outcomes.

#### 6. Scratch wound cell migration assay (wound healing assay)

To generate wounds on breast cancer cells, IBIDI culture inserts (IBIDI, Martinsried, Germany) were placed into wells of 24-well plates, and cells were added into the two reservoirs of the inserts. Cells were plated at  $1.5 \times 10^5$  BT474 cells/well,  $1.2 \times 10^5$  MCF7 cells/well,  $4 \times 10^4$  MDA-MB-231 cells/well, and  $3 \times 10^4$  Hs 578T cells/well. After incubation for 24 hr in a 37°C CO<sub>2</sub> incubator, the culture inserts were removed, and images of the

wounds were taken at 0 and 24 hr using an Olympus IX71 microscope (Olympus France; Rungis, France).

For kinetic analysis, cIAP2-silenced MDA-MB-231 cells ( $4 \times 10^4$  cells/well) and Hs 578T ( $1 \times 10^4$  cells/well) were seeded into a 96-well Essen ImageLock microplate (Essen BioScience, Ann Arbor, MI, USA) and incubated in a 37°C CO<sub>2</sub> incubator for 24 hr. The cell monolayer was scratched using a 96-well Wound Maker and then washed with phosphate-buffered saline (PBS) to remove the detached cells. Images of the wounds were automatically recorded at 6hr intervals for 24 hr using IncuCyte ZOOM software.

#### 7. Scratch wound cell invasion assays

cIAP2-silenced MDA-MB-231 cells ( $4 \times 10^4$  cells/well) and Hs 578T cells ( $1 \times 10^4$  cells/well) were seeded into a 96-well Essen ImageLock microplate coated with diluted Matrigel (100 µg/mL) in cold culture medium, and cells were then incubated in a 37°C CO<sub>2</sub> incubator for 24 hr. The cells were scratched using a 96-well Wound Maker and then washed with PBS to remove detached cells. Next, 50 µL diluted Matrigel (8 mg/mL) in cold culture medium was added to each well, and cells were incubated in a 37°C CO<sub>2</sub> incubator for 30 min. When the Matrigel solution was solidified, 100 µL cell culture medium was added to each well. Images of the wounds were automatically recorded at 6 hr intervals for 48 hr using IncuCyte ZOOM software.

#### 8. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR

RNA was extracted using TRIZol reagent (Invitrogen), and cDNA was synthesized using

Oligo dT primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Synthesized cDNAs were used as templates for subsequent PCR of BIRC1, BIRC2, BIRC3, BIRC4, BIRC5, BIRC6, BIRC7, BIRC8, CDH1, CDH2, EPCAM, VIM, ACTA2, SNAI1, SNAI2, and GAPDH genes.

The following primers were used: *CDH1*, 5'-GTCAGTTCAGACTCCAGCCC-3', 5'-AAATTCCTCTGCCCAGGACG-3'; *EPCAM*, 5'-CTCCAGAACAATGATGGGCT-3', 5'-CCAGTAGGTTCTCACTCGCTC-3'; *CDH2*, 5'-GGTGGAGGAGAAGAAGACCAG-3', 5'-GGCATCAGGCTCCACAGT-3'; *Vim*, 5'-GTTTCCAAGCCTGACCTCAC-3', 5'-GCTTCAACGGCAAAGTTCTC-3'; *ACTA2*, 5'-GTGAAGAAGAGGACAGCACTG-3', 5'-CACATACATGGCTGGGACATTG-3'; *SNAI2*, 5'-CAAGGACACATTAGAACTCAC-3', 5'-GTGCAGGAGAGACATTCTGGA-3'; *SNAI1*, 5'-ACTTCAGTCTCTTCCTTGGAG-3', 5'-TGACATCTGAGTGGGTCTGG-3'; *BIRC1*, 5'-CGTCCCATTGTTGCCAGT-3', 5'-GATCAGTTTGGCCACTCG-3'; *BIRC2*, 5'-GTGGTGGGAAGCTCAGTAAC-3', 5'-CATCATCATTGCGACCCACA-3'; *BIRC3*, 5'-AATGCTTTTGCTGTGATGGTG-3', 5'-GCTTGAACCTGACGGATGAAC-3'; *BIRC4*, 5'-GGGGTTCAGTTTCAAGGACAT-3', 5'-CCACAAGGAACAAAAACGATAG-3'; *BIRC5*, 5'-GACGACCCCATAGAGGAACAT-3', 5'-GCCAGAGGCCTCAATCCAT-3'; *BIRC6*, 5'-ATGTGCTTCCAACCCTTCCT-3', 5'-GGCTCAGTTTGATTCACGC-3'; *BIRC7*, 5'-TTCTTCCACACAGGCCATCA-3', 5'-CTGTGGACAAAGTCTCTTCC-3'; *BIRC8*, 5'-GAGACGGTGGACAAGTCCTA-3', 5'-TTGCCACCTGTCTACCGCTT-3'; *GAPDH*, 5'-GATGGCATGGACTGTGGTCA-3', 5'-GCAATGCCTCCTGCACCACC-3'.

Quantitative real-time RT-PCR was carried out using a KAPA SYBR FAST qPCR kit (KaPa Biosystems, Wilmington, MA, USA), and amplification was performed on an ABI

Prism 7000 detection system (Applied Biosystems, Waltham, MA, USA) according to the conditions recommended by the manufacturer. The experiments were performed in triplicate and normalized to the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). The relative expression levels of the target genes were calculated by the  $2^{-\Delta\Delta C_t}$  method.

#### 9. Statistical methods

Unpaired Student's t-tests were used to determine significant differences between groups. All statistical analyses were performed using GraphPad Prism 6.0a software (GraphPad Software, La Jolla, CA, USA). Values represent means  $\pm$  standard deviations (SDs). Differences with P values of less than 0.05 were considered significant.

### III. RESULT

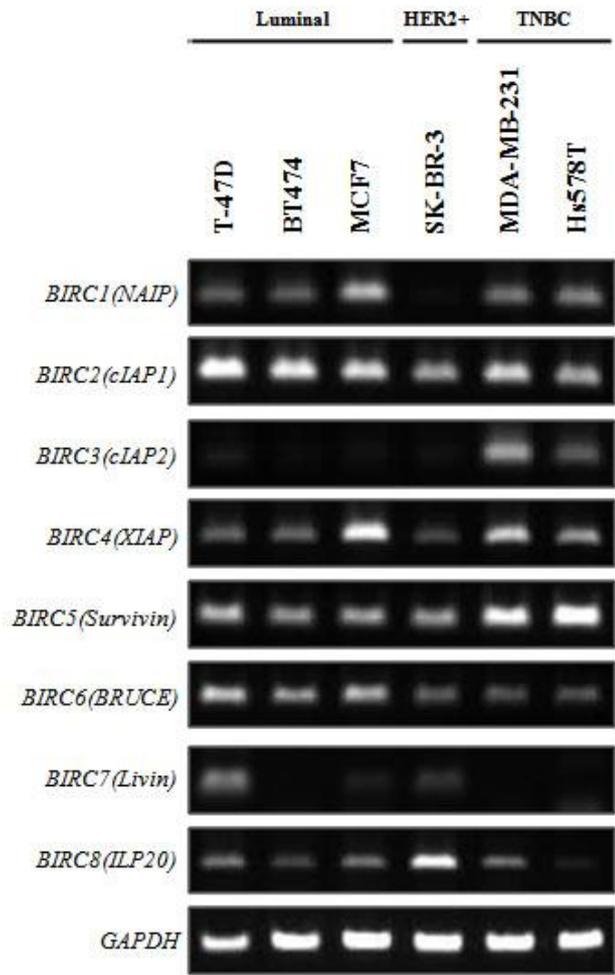
#### 1. Expression of cIAP2 in various breast cancer cell lines

To investigate the relevance of IAP expression in TNBC, I assessed mRNA expression levels for all IAP members in luminal-type (T-47D, BT474, MCF7), HER2-positive-type (SK-BR-3), and triple-negative (MDA-MB-231 and Hs 578T) breast cancer cell lines (Table 1).<sup>19-21</sup> Among the eight IAP genes evaluated in this study (BIRC1–8), only cIAP2 (BIRC3) was highly expressed in TNBC cells compared with that in other breast cancer subtypes, while other IAP members were detected in various breast cancer cells, irrespective of their subtypes (Fig. 1). And the expression level of BIRC7-8 was relatively low compared to other IAP members.

**Table1. Characteristics of the breast cancer cell lines used in this study.**

Cell line	ER	PR	HER2	Subtype
MCF7	+	+	-	Luminal <sup>[19-21]</sup>
T-47D	+	+	-	Luminal <sup>[19-21]</sup>
BT474	+	+	+	Luminal <sup>[19-21]</sup>
SK-BR-3	-	-	+	HER2-positive <sup>[19-21]</sup>
MDA-MB-231	-	-	-	Triple negative <sup>[19-21]</sup>
Hs578T	-	-	-	Triple negative <sup>[19-21]</sup>

Relevant characteristics of breast cancer cell lines used in this study were summarized here. Status of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2(HER2)/neu and breast cancer subtypes were indicated based on previously published data



**Fig. 1. Analysis of IAP expression in breast cancer cells.** mRNA expression of IAPs was examined in luminal-type breast cancer cells (T-47D, BT474, MCF7), HER2-positive breast cancer cells (SK-BR-3), and triple-negative breast cancer cells (MDA-MB-231, Hs 578T).

## 2. Analysis of cIAP2 expression in TNBC tissues

To determine the expression level of cIAP2 in breast cancers tissues, I performed TMA analysis using IHC staining. For interpretation of the IHC stain results, the IHC tests for cIAP2 was categorized as negative (0), weak/moderate (1+), strong (2+) in high-power fields (200x magnification) according to the intensity of cytoplasmic staining (Fig. 2) and using quantitative scoring methods (Table 2).



**Fig. 2. Immunohistochemical analysis of cIAP2 in triple-negative breast cancer tissues.** Expression of cIAP2 in triple-negative breast cancer tissue was evaluated in high-power fields (200 $\times$ ). (A) Negative for cIAP2. (B) 1+ for cIAP2. (C) 2+ for cIAP2.

**Table 2. Quick score for cIAP2**

<b>% Staining score</b>	<b>% Nuclei staining</b>
0	0%
1	1-4%
2	5-9%
3	10-19%
4	>20%
5	>50%

<b>Intensity score</b>	<b>Staining intensity</b>
0	no staining
1	weak staining
2	moderate staining
3	strong staining

Quick score=% Staining score + Intensity score  
 Giving a range from 0-8

About 44% of patients with TNBC showed high expression of cIAP2 and about 52% of patients with TNBC showed weak/moderate expression of cIAP2. To sum up, about 96% of patients with TNBC showed positive expression of cIAP2 in cancer tissues, whereas only 4% of TNBC tissues showed no cIAP2 expression (Table 3) suggesting that cIAP2 may play a role in TNBC.

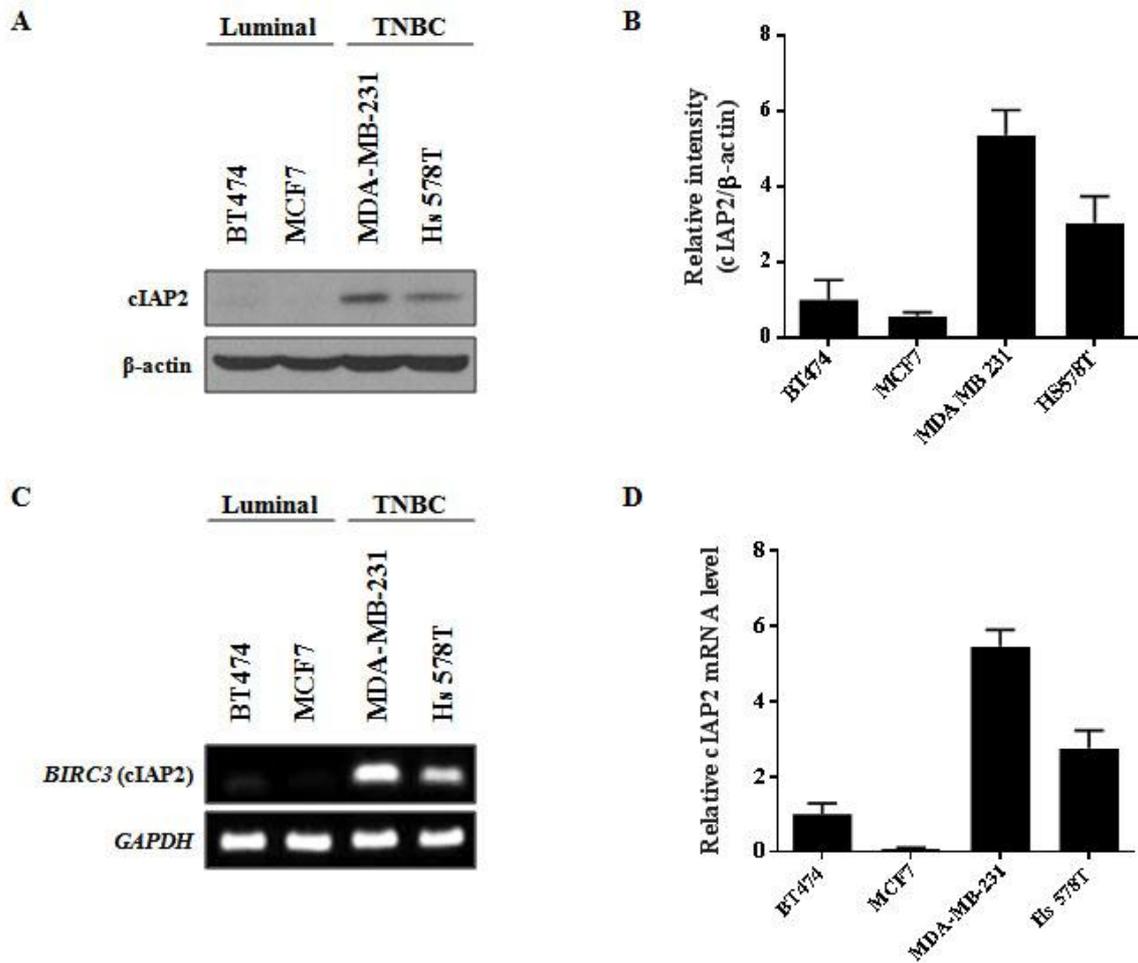
**Table 3. Comparison of cIAP2 expression in triple-negative breast cancer tissue microarrays using immunohistochemistry (IHC).**

Interpretation	TNBC TMA (N=100)		
	Quick score	Frequency	Percent
<b>Negative (0)</b>	0	4	
	1	0	<b>4%</b>
	2	0	
<b>Weak/Moderate (1+)</b>	3	20	
	4	13	<b>52%</b>
	5	19	
<b>Strong (2+)</b>	6	38	
	7	6	<b>44%</b>
	8	0	

The cIAP2-positive status was assigned for scores “1+” and “2+”.

### 3. Analysis of cIAP2 expression in luminal-type breast cancer cells and TNBC cells.

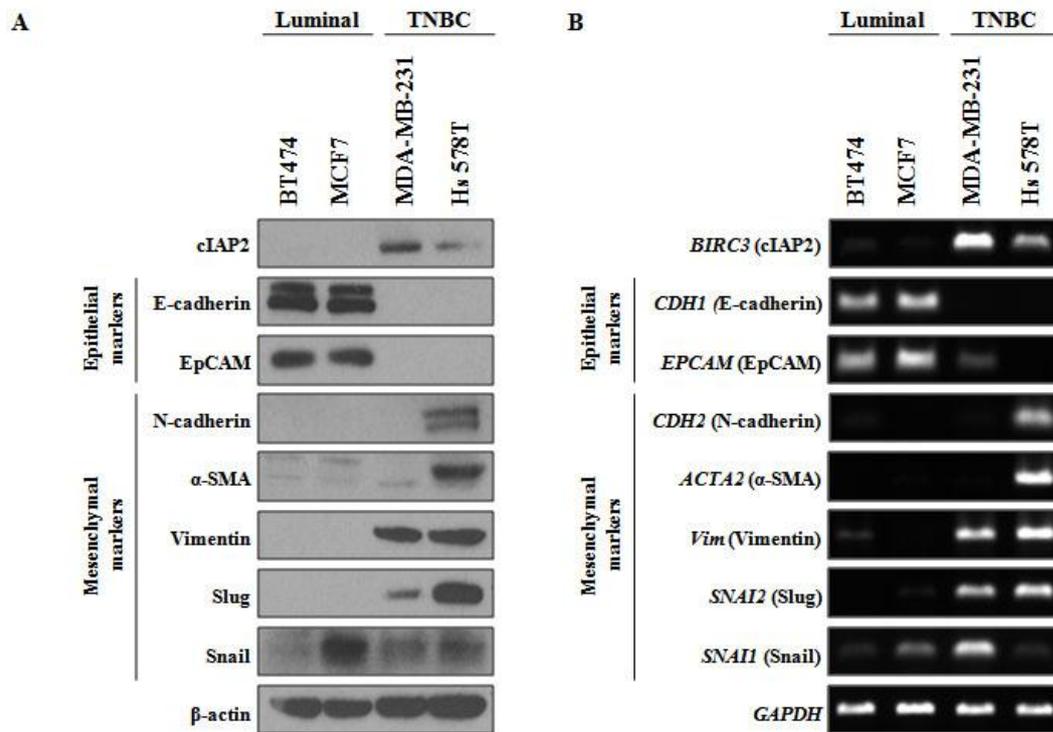
To confirm the molecular meaning of the TMA results, I assessed mRNA and protein levels of cIAP2 in luminal-type breast cancer cell lines (BT474 and MCF7; non-TNBC) and TNBC cell lines (MDA-MB-231 and Hs 578T). In accordance with IHC analysis of breast cancer TMA in Table 1, cIAP2 was highly expressed in TNBC cell lines but barely detected in luminal-type cell lines at both the protein (Fig. 3A and 3B) and mRNA (Fig. 3C and 3D) levels.



**Fig. 3. cIAP2 expression in breast cancer cell lines.** Expression of cIAP2 was examined on luminal-type (BT474, MCF7) and triple-negative (MDA-MB-231, Hs 578T) breast cancer cells. (A) Western blotting was performed to determine the protein expression level of cIAP2.  $\beta$ -Actin was used as a loading control. (B) Graphical representation of western blot band intensities quantified using Image J (n = 3). Expression of cIAP2 mRNA was examined by (C) RT-PCR and (D) quantitative real-time PCR. For quantification of the results in (B) and (D), the cIAP2 protein and mRNA level for BT474 cells was set to 1. Values represent the means  $\pm$  standard deviations.

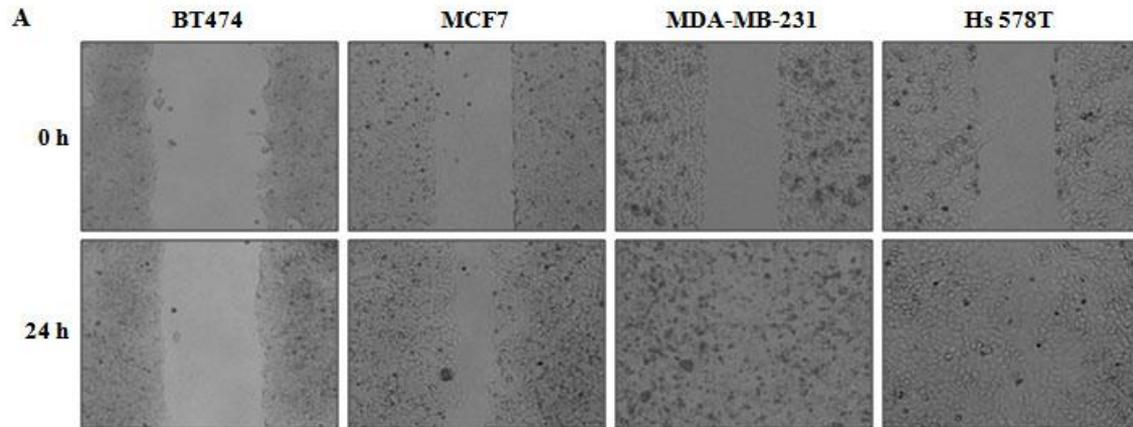
#### 4. Analysis of cIAP2 and EMT-associated gene expression in breast cancer cells

These data for TMA, qPCR, and IHC analyses showed strong correlations between cIAP2 expression and TNBC. Thus, I hypothesized that cIAP2 may play important roles in regulating the clinical features of TNBCs, including poor prognosis and high metastatic tendency. Therefore, I investigated the possibility that cIAP2 may regulate the metastatic characteristics of breast cancers by detection of epithelial/mesenchymal markers known to be closely related to metastatic progression. At both the protein and mRNA levels, epithelial markers including E-Cadherin, EpCAM were only detected in luminal-type cell lines; in contrast, mesenchymal markers including N-cadherin,  $\alpha$ -SMA, Vimentin, Slug were primarily expressed in TNBC cell lines. In TNBC cells, epithelial markers including E-Cadherin, EpCAM was not detected at protein level (Fig. 4A and 4B).



**Fig. 4. Comparison of cIAP2 expression and EMT-associated molecules in breast cancer cells.** Expression of cIAP2 and EMT-associated molecules were examined in luminal-type (BT474, MCF7) and triple-negative (MDA-MB-231, Hs 578T) breast cancer cells at (A) protein and (B) mRNA levels.  $\beta$ -Actin and GAPDH were used as loading controls.

Next, wound healing assays were performed to determine the effects of cIAP2 expression on the metastatic phenotype. The results showed far higher migration capacity in TNBC cell lines compare with that in luminal-type cell lines (Fig. 5).



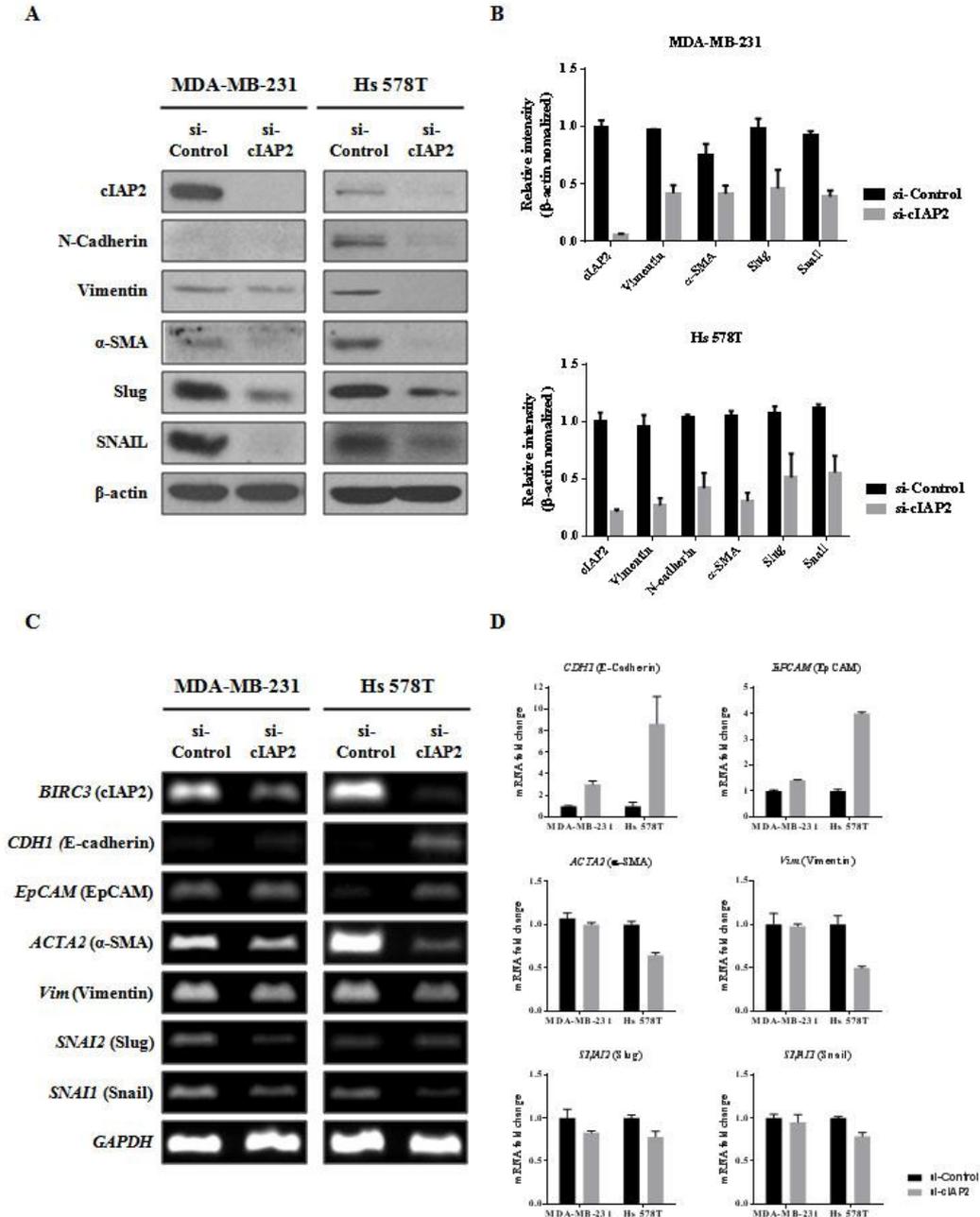
**Fig. 5. Cell motility in various breast cancer cell lines (BT474, MCF7, MDA-MB-231, Hs 578T).** Cell migration capacity was examined by wound healing assays. Images were taken at 0 and 24 hr. The wound width was 500  $\mu\text{m}$  (10 $\times$  magnification).

#### 5. Effects of EMT-associated gene expression on cIAP2 silencing in TNBC cells

Based on epithelial/mesenchymal marker expression patterns and migratory capacity (Fig. 4 and Fig. 5), I speculated that cIAP2 may function as a regulator of the EMT. To verify this, I evaluated changes in epithelial and mesenchymal markers after silencing of cIAP2 mRNA in TNBC cell lines. TNBC cells were transfected with cIAP2-siRNA or control-siRNA. After 72 hours, cells were harvested. The optimal concentrations of siRNA for gene silencing were 50 nM for MDA-MB-231 cells and 25 nM for Hs 578T cells.

Notably, all of the mesenchymal markers including N-cadherin,  $\alpha$ -SMA, Vimentin, Slug, Snail were reduced by cIAP2 knockdown at both the protein and mRNA levels (Fig. 6A–6C). In contrast, mRNA of epithelial markers, including CDH1 (encoding E-cadherin) and EPCAM (encoding EpCAM) were obviously increased in cIAP2-knockdown cells (Fig. 6C

and 6D). But expression of epithelial markers including E-cadherin and EpCAM were not detected in cIAP2-knockdown TNBC cells at protein level (data not shown). These changes in mesenchymal to epithelial markers by cIAP2 knockdown indicated that cIAP2 was a positive regulator of the EMT process.



**Fig 6. Analysis of EMT-associated molecules in TNBC cells (MDA-MB-231 and Hs 578T) with cIAP2 silencing.** (A) Expression of EMT-associated mesenchymal markers, including N-cadherin, vimentin,  $\alpha$ -SMA, Snail, and Slug, was determined at the protein level in MDA-MB-231 and Hs 578T cells transfected with si-cIAP2.  $\beta$ -Actin was used as a loading control. (B) Graphical representation of western blot band intensities quantified using Image

J (n = 3). (C and D) Expression of EMT-associated markers, including CDH1, EPCAM, ACTA2, Vim, SNAI1, and SNAI2, was determined at the mRNA level in MDA-MB-231 and Hs 578T cells transfected with si-cIAP2 by (C) RT-PCR and (D) quantitative real-time PCR. Values represent the means  $\pm$  standard deviations.

#### 6. Analysis of cell migration and invasion ability in cIAP2-knockdown TNBC cells

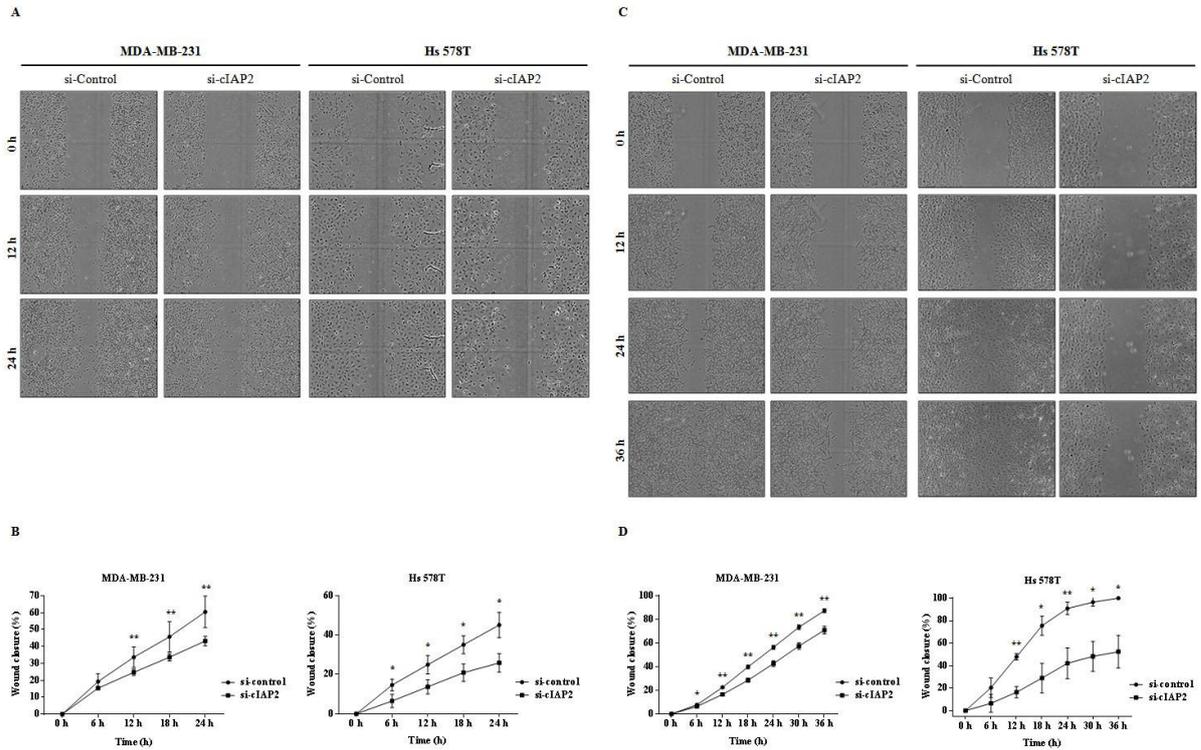
During the EMT process, changes in epithelial/mesenchymal features of cells lead to migration and invasion, which are essential for cancer metastasis<sup>22</sup>. As mentioned above, I found that silencing of cIAP2 induces MET-like process in TNBC cells. Thus, I assessed the migration and invasion capacities of cIAP2-knockdown TNBC cells. cIAP2-silenced TNBC cells were seeded into a 96-well plate and incubated in a 37°C CO<sub>2</sub> incubator for 24 hr. The cell monolayer was scratched using a 96-well Wound Maker and then washed with phosphate-buffered saline (PBS) to remove the detached cells.

In wound healing assays, control MDA-MB-231 and Hs 578T cells showed high migratory capacity, consistent with the results shown in Figure 5; however, significantly attenuated cell migration was observed in both cell lines with cIAP2 knockdown (Fig. 7A and 7B). And difference of cell migration ability between si-control and si-cIAP2 in TNBC cells was high at 24 hours.

The wounds were then filled with Matrigel to mimic the extracellular matrix in order to assess invasiveness. cIAP2-silenced TNBC cells were seeded into a 96-well plate coated with diluted Matrigel in cold culture medium, and cells were then incubated in a 37°C CO<sub>2</sub>

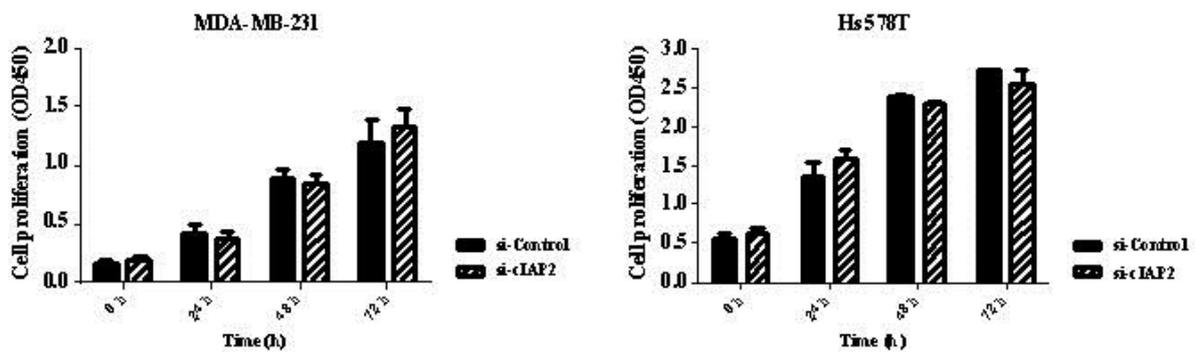
incubator for 24 hr. The cells were scratched using a 96-well Wound Maker and then washed with PBS to remove detached cells. Next, diluted Matrigel (8 mg/mL) in cold culture medium was added to each well, and cells were incubated in a 37°C CO<sub>2</sub> incubator for 30 min. When the Matrigel solution was solidified, cell culture medium was added to each well.

Analysis showed that invasiveness was greatly inhibited in cIAP2-knockdown cells (Fig. 7C and 7D). And difference of cell invasiveness between si-control and si-cIAP2 in TNBC cells was high at 36 hours. I suppose that it takes more time to break through solidified matrigel. Thus, cIAP2 played critical roles in determining the cell migration and invasion capacity in TNBC cells.



**Fig. 7. Silencing of cIAP2 suppressed the migration and invasion ability of TNBC cells (MDA-MB-231 and Hs 578T).** (A) Migration of MDA-MB-231 (si-control and si-cIAP2) and Hs 578T (si-control and si-cIAP2) cells was examined by wound healing assay. (C) Invasion capacity of MDA-MB-231 (si-control and si-cIAP2) and Hs 578T (si-control and si-cIAP2) cells was examined by scratch wound invasion assay. The wound gap was photographed every 6 hr. The initial wound width was 700  $\mu\text{m}$  (10 $\times$  magnification). (B and D) Wound closure (%) was measured using IncuCyte ZOOM software (n = 5). Values represent the means  $\pm$  standard deviations. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

One of the molecular functions of cIAP2 is inhibition of cell death<sup>11</sup>, which may affect cell migration and invasion. However, among IAPs, the major inhibitory molecule of apoptosis is thought to be XIAP, and cIAP2 may have a relatively minor role in the regulation of apoptosis, despite its binding affinity to caspase.<sup>23,24</sup> To exclude the effects of cIAP2 on cell death in measurement of cell migration and invasion, I also evaluated cell proliferation after cIAP2 knockdown; no significant difference was observed between si-scramble- and si-cIAP2-transfected cells (Fig. 8). Thus, the reduced migratory/invasive capacity of cIAP2-knockdown cells was assumed to be mostly due to inhibition of the EMT.



**Fig. 8. Effect of cIAP2 silencing on cell proliferation in two TNBC cell lines (MDA-MB-231 and Hs 578T).** Cell proliferation rates of the indicated cell lines were measured using a Cell Counting Kit-8 (CCK-8). Values are represented as means  $\pm$  standard deviations.

## 7. Analysis of the AKT signaling pathway in cIAP2-mediated induction of the EMT

Next, I attempted to investigate the molecular mechanisms of cIAP2-mediated EMT induction. Several signaling pathways related to the EMT process were evaluated (data not shown), and I found that the AKT signaling pathway was significantly altered following cIAP2 manipulation (Fig. 9). In previous reports, the AKT signaling pathway was shown to induce the EMT process through inhibitory phosphorylation of GSK3 $\beta$ , a negative regulator of the EMT-related transcription factor Snail.<sup>25,26</sup> Thus, I evaluated the expression of AKT-associated signaling molecules in cIAP2-knockdown TNBC cells. Western blot analysis showed that phosphorylation of AKT was attenuated by silencing of cIAP2, leading to reduced phosphorylation of GSK3 $\beta$  and subsequent destabilization of Snail (Fig. 9A). As a consequence of reduced GSK3 $\beta$  phosphorylation, I observed reduced expression of Snail at the protein level (Fig. 6A and 6B). And I also observed reduced expression of Slug which is known as one of the transcription factors of the EMT (data not shown).

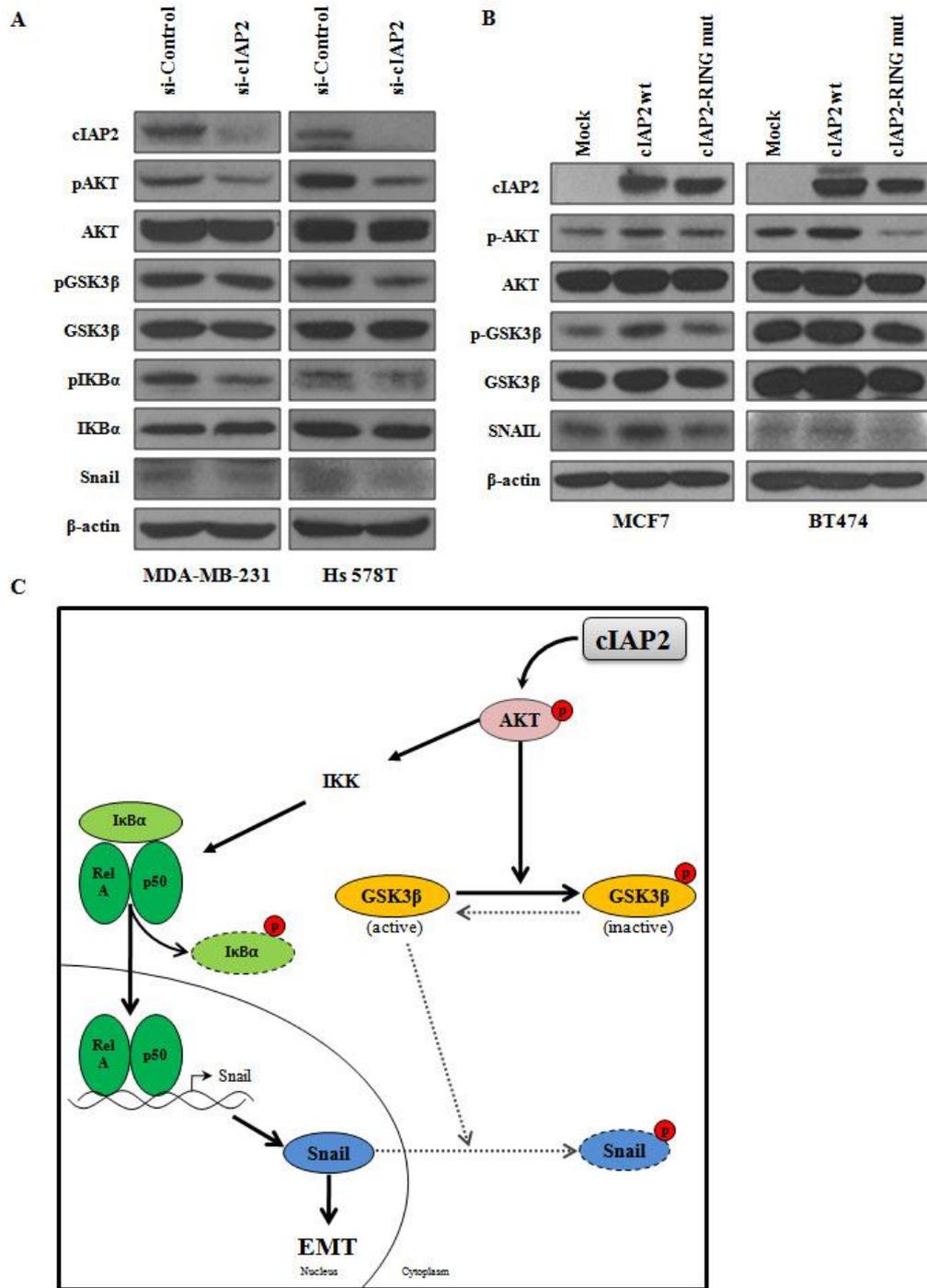
Interestingly, SNAI1 expression was also decreased by silencing of cIAP2 (Fig. 6C and 6D). Nuclear factor kappa B (NF- $\kappa$ B) is a well-known transactivator of SNAI1<sup>27</sup> and a target of AKT regulation<sup>28</sup>. Therefore, I evaluated the phosphorylation of I $\kappa$ B $\alpha$ , which acts as an inhibitor of NF- $\kappa$ B activation. In western blot analysis, I found reduced phosphorylation of I $\kappa$ B $\alpha$  by silencing of cIAP2 (Fig. 9A).

Additionally, I analyzed the expression of AKT-associated signaling molecules in cIAP2-transfected MCF7 and BT474 cells. Cells were transfected with PRK5-flag, PRK5-cIAP2 wild type-flag and PRK5-cIAP2 RING domain mutant (K558/559V)-flag plasmid.

After 72 hours, cells were harvested. Western blot analysis showed that phosphorylation of

AKT was increased by ectopic expression of wild-type cIAP2, leading to increased GSK3 $\beta$  phosphorylation and subsequent stabilization of Snail protein (Fig. 7B). But ectopic expression of RING domain mutant cIAP2 had no effect on AKT signaling pathway. So I suggest that RING domain of cIAP2 which function as E3 ligases is important for inducing AKT signaling pathway.

Thus, these data collectively suggested that cIAP2 induced the EMT through activation of both AKT/GSK3 $\beta$  and AKT/NF- $\kappa$ B signaling pathways (Fig. 7C).



**Fig. 9. cIAP2 promoted the EMT through the AKT signaling pathway.** The expression of AKT signaling pathway-associated molecules was analyzed in (A) cIAP2-silenced TNBC cells and (B) luminal-type breast cancer cells ectopically expressing cIAP2.  $\beta$ -Actin was used

as a loading control. (C) Proposed model for the molecular mechanisms through which cIAP2 induced the EMT. The bold lines represented an effect that was reinforced by cIAP2, whereas the dotted lines represented an effect that was weakened by cIAP2.

#### IV. DISCUSSION

IAPs are proto-oncogenes involved in the regulation of tumor progression, metastasis, cell survival, and migration. In particular, cIAP2 is upregulated in colorectal cancer, mucosa associated lymphoid tissue lymphoma, acute myeloid leukemia, and lung cancer and is associated with poor tumor prognosis.<sup>10</sup> However, no reports have described the clinical significance of cIAP2 in breast cancers.

Although cIAP2 has been reported to be upregulated in several types of cancer, there are controversies in IAP's role which is positive or negative for EMT processes.<sup>14,15,17,29,30</sup> IAPs share high homology and show functional redundancy; however, each IAP may induce different effects in EMT regulation. In the current study, these findings supported that cIAP2 functioned as a positive regulator of the EMT in TNBC. Despite these findings, it is still unclear whether other IAPs also promote the EMT process, although their expression levels did not differ among cell lines showing low and high migratory capacity.

In this study, I verified that cIAP2, which was up-regulated in TNBC compared with other breast cancer subtypes, induced the EMT in TNBC cells via activation of the AKT signaling pathway. And I found that E3 ligase activity of cIAP2 RING domain is important for inducing AKT signaling pathway. So it was hypothesized that cIAP2 might ubiquitinate and lead to degradation of certain molecule which repress PI3K-AKT signaling such as PTEN<sup>31</sup>. There might be other targets of E3 ligase activity of cIAP2 responsible for inducing AKT signaling pathway.

Several reports of IAP-mediated EMT induction have shown that this mechanism involves the regulation of small Rho GTPases.<sup>14,16,17,32,33</sup> Gradients of Rho GTPase proteins are

associated with cell polarity and mobility, which are critical factors involved in the EMT process.<sup>34</sup> Because the EMT process involves various cellular changes, adjustment of major transcription factors, such as Snail, Twist, and Zeb family proteins, is also necessary. Accordingly, AKT signaling and Snail regulation by cIAP2 could be the main mechanism mediating the EMT process.

The AKT signaling pathway has been shown to positively influence EMT progression.<sup>35</sup> The mechanisms of AKT-mediated EMT induction can be summarized as follows. First, activated AKT phosphorylates GSK3 $\beta$  (inhibitory phosphorylation) and subsequently prohibits the phosphorylation of Snail, leading to its degradation.<sup>26</sup> Second, activated AKT can also phosphorylate and activate IKK, which triggers nuclear localization of NF- $\kappa$ B. Then, NF- $\kappa$ B activates the transcription of SNAI1.<sup>36</sup> Consistent with these findings, I observed reduced Snail expression at both the protein and mRNA level, following cIAP2 knockdown, suggesting that cIAP2 induced the EMT by both transcriptional and post-translational regulation of Snail through induction of AKT signaling.

Slug is another transcription factor with important roles in the EMT process.<sup>37</sup> In this study, I observed changes in SNAI2 (Slug) as well as SNAI1 (Snail) at both the mRNA and protein levels after cIAP2 manipulation, suggesting the possibility of Slug regulation by AKT signaling, similar to the mechanism described for Snail regulation. Indeed, post-translational regulation of Slug by AKT/GSK3 $\beta$  signaling has recently been described.<sup>37</sup> Additionally, some reports have shown that Slug is regulated by the NF- $\kappa$ B signaling pathway.<sup>38,39</sup> However, the roles of AKT in regulation of SNAI2 have not yet been clarified. Further studies are needed to better establish the roles of the AKT signaling pathway in the EMT process.

The NF- $\kappa$ B signaling pathway is involved in many biological processes, including cell proliferation, cell differentiation, immune responses, and apoptosis, and the dysregulation of NF- $\kappa$ B is involved in many diseases, including inflammatory/autoimmune disease, tissue malformation, and tumorigenesis.<sup>40</sup> cIAP1 and cIAP2 have been reported to be essential for NF- $\kappa$ B signal transduction via K63-linked ubiquitination of RIP1.<sup>11,12</sup> At the same time, cIAP1 and cIAP2 are also targets of NF- $\kappa$ B-transactivation, suggesting the involvement of positive feedback in the regulation of cIAP1/2 expression.<sup>41</sup> As shown in the study, cIAP2 activated AKT signaling, supporting the involvement of another feedback loop in the regulation of cIAP1/2 expression as AKT signaling can also activate NF- $\kappa$ B. Although more in-depth studies are required, these results provided insights into a novel signaling pathway and could improve understanding of NF- $\kappa$ B signal transduction.

I observed clear and significant effect on the EMT processes by cIAP2 knockdown in cIAP2 positive TNBC cell lines. However, ectopic-expression of cIAP2 in breast cancer cell lines which rarely express cIAP2 did not show meaningful changes on EMT processes, except activation of AKT signaling pathway (data not shown). This may be due to the masking effect of ER and/or PR in breast cancer cells. In coincidence with these experiment, most of cIAP2 negative breast cancer cell lines are luminal type (expressing ER and/or PR), and some reports suggest that ER and/or PR repress EMT progression<sup>42-45</sup> in spite of controversy.<sup>46,47</sup> Therefore, it is possible that EMT progression is eventually prohibited by ER and/or PR signaling in cIAP2-negative breast cancer cells despite ectopic expression of cIAP2. To reveal the exact mechanisms of this, more in depth study would be required.

Recently, several small molecule inhibitors of IAPs (including diablo IAP-binding mitochondrial protein-mimicking molecules [SMAC mimetics]) have been developed, some

of which have entered phase II trials as potential cancer therapies.<sup>48</sup> The major evidence supporting the use of SMAC mimetics is induction of apoptosis by inhibition of IAPs, which can drive cancer cell death. Thus, based on these findings, it is possible that SMAC mimetics also attenuate EMT progression via inhibition of cIAP2, eventually acting to block cancer metastasis. Therefore, SMAC mimetics, as therapeutics for both cell death induction and EMT inhibition, may be novel candidates for the treatment of TNBC.

## V. CONCLUSION

These current findings revealed that cIAP2 was highly expressed in TNBC compared with other types of breast cancer, both in vivo and in vitro. Additionally, I found that cIAP2 regulated the EMT via the AKT signaling pathway. And I suggest that RING domain of cIAP2 may have critical roles in AKT signaling pathway. These data suggested that cIAP2 could be an attractive candidate for development of targeted therapeutics and a potential diagnostic marker for predicting tumor metastasis.

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## ABSTRACT (IN KOREAN)

유방암 세포의 상피간엽이행(EMT)에서 cIAP2의 역할 규명

<지도교수 이재면>

연세대학교 대학원 의과학과

조수지

유방암은 에스트로겐 수용체(estrogen receptor), 프로게스테론 수용체(progesterone receptor), human epidermal growth factor receptor(HER2/neu)의 발현 여부로 분류된다. 위의 3가지 수용체를 모두 발현하지 않는 삼중음성유방암(triple negative breast cancer, TNBC)은 유방암의 다른 아형에 비해 예후가 나쁘고 재발률이 높으며 암세포의 전이가 빠르다.

삼중음성유방암의 초기 암세포는 상피간엽이행(Epithelial to mesenchymal transition; EMT)을 통해 상피세포가 간엽세포의 모양과 특징으로 변하면서 줄기세포의 특성을 획득하게 되고 이로 인해 전이가 가속화된다. 세포 내/외부 신호의 상호작용에 의해 상피간엽이행이 유도되면 세포 내부에서는 SNAIL, SNAIL2, ZEB, TWIST 등의 전사인자가 활성화 되며, 이들 전사제어인자의 작용에 의해 앞서 언급된 상피세포/간엽세포 연관 인자들의 발현이 조절된다.

이러한 일련의 신호 전달과정은 다양한 인자들의 상호작용에 의해 진행되므로, 이의 조절을 담당하는 중간조절인자의 역할은 상피간엽이행과정에서 중요한 부분을 차지한다. 최근 관련 연구가 활발히 진행되면서, LOXL2, IAPs 등 상피간엽이행 과정의 중간조절인자들에 대한 새로운 역할이 제시되고 있다.

IAPs(Inhibitor of apoptosis proteins)는 caspase 3,7,9를 억제하여 세포 사멸을 조절하는 기능을 하며 NAIP, cIAP1/2, XIAP, Survivin, Apollon, ML-IAP, ILP2 등으로 구성되어 있다. 최근에는 IAPs가 세포 사멸을 조절하는 기능 이외에도 세포 분화나 운동성, 세포 이동, 침습, 암 전이에 관여 할 것이라는 보고가 증가하고 있다. 다양한 암에서 IAP의 mRNA 및 단백질의 발현이 증가되어 있다는 것이 보고되었지만, 암세포에서 IAP가 종양 형성 및 전이에 미치는 영향에는 상반된 의견이 존재한다. 또한 분자적 기적 연구에서도 IAPs의 기능에 관한 논란이 있는 상황이므로, IAP와 암 전이 사이의 연관관계 및 관련 기전의 규명이 필요하다.

본 연구에서는 IAP 중 하나인 cIAP2가 삼중음성유방암 세포 및 조직에서 특이적으로 발현되는 것을 확인하였다. 이러한 결과를 바탕으로 삼중음성유방암 세포에서 cIAP2의 유전자 발현을 억제하였을 때 상피간엽이행 과정이 억제되었고, AKT 신호전달 기전 억제와 Snail의 발현 조절을 통해 세포의 이동성 및 침습성이 감소하는 것을 관찰하였다. 또한 cIAP2의 발현을 통해 AKT 신호전달 기전이 활성화 되었고, 이 과정에서 cIAP2의 RING domain이 중요하다는 것을 확인하였다.

본 연구에서 삼중음성유방암에서 cIAP2가 AKT 신호전달 기전을 통해 상피간엽이행 과정을 조절한다는 것을 밝혔고, 삼중음성유방암의 바이오마커로서 cIAP2의 가능성을 제시했다.

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핵심되는 말: 삼중음성유방암(triple negative breast cancer, TNBC), cIAP2, 상피간엽이행(Epithelial to mesenchymal transition; EMT), AKT 신호전달