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A Master's Thesis

Baicalein induces ubiquitylation of Snail and Slug via CYR61 signaling pathway in human breast cancer cells

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Graduate School

Jeju National University

December, 2014

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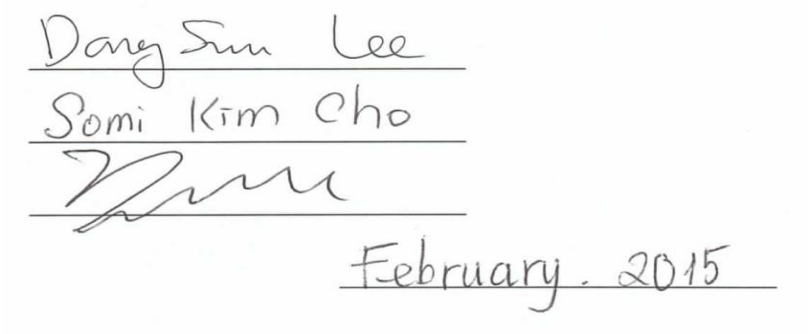
(Supervised by Professor Somi Kim)

A thesis submitted in partial fulfillment of the
requirement for the degree of Master of Biotechnology

2015.02

This thesis has been examined and approved.



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Graduate School

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Abstract

Epithelial-mesenchymal transition (EMT), a critical step in the acquisition of metastatic state, is an attractive target for therapeutic interventions directed against tumor metastasis. Baicalein is a widely used herbal medicine that has been used historically in anti-inflammatory and anti-cancer therapy. The purpose of this study was to determine a molecular mechanism of Baicalein anti-metastatic activity which remains poorly understood and warrants further investigations. Cysteine-rich protein 61 (CCN1/CYR61) has been implicated as an important mediator in proliferation and metastasis of breast cancer, which indicated that blockage of CYR61 might be a potent target for breast cancer treatment. Here we showed the novel mechanism in which Baicalein inhibits EMT by targeting CYR61 and then Akt/GSK3β signaling pathway. Subsequently, Baicalein induced the degradation and ubiquytilation of Snail and Slug through GSK3β activation. Interestingly, Baicalein inhibited the direct interaction of LOXL-2 with Snail and Slug that attenuate GSK3β-dependent Snail and Slug degradation. Our findings provide new insights into the anti-metastasis of Baicalein and may contribute to beneficial use of Baicalein in breast cancer therapies.

요약문

Epithelial-mesenchymal transition (EMT)는 전이상태를 획득하기 위해 중요한 단계이며, 암 전이를 치료하기 위한 표적이 된다. Baicalein 은 항염증과 항암치료에 있어서 예로부터 널리 사용된 천연 의약품이다. 본 연구는 아직 연구가 미흡한 Baicalein 의 항전이 활성의 분자적 메커니즘을 구명하고자 하였다. Cysteine-rich protein 61 (CCN1/CYR61)은 유방암의 증식과 전이에 관련된 중요한 매개 단백질이므로, 유방암 치료에 있어서 CYR61 을 억제하는 것은 중요한 표적이 될 수 있다. 본 연구에서는 Baicalein 이 CYR61 과 Akt/GSK3β pathway 를 표적함으로써 EMT 를 억제하는 메커니즘을 밝혔다. Baicalein 은 GSK3β 의 활성을 통해 Snail 과 Slug 분해와 유비퀴틴화를 유도하였다. 또한, Baicalein 은 GSK3β 에 의한 Snail 과 Slug 의 분해를 억제하는 LOXL-2 와 Snail, Slug 의 상호작용을 직접적으로 저해하는 것으로 나타났다. 따라서 이러한 결과들은 Baicalein 의 항전이 활성에 대한 새로운 이해를 제공함으로써 유방암 치료에 있어서 Baicalein 의 유용한 사용에 기여할 수 있을 것으로 생각된다.

1. Introduction

Epidemiological studies now indicate that an increased intake of dietary flavonoids is associated with a decreased risk of inflammation, hypertension, cardiovascular disease, and bacterial and viral infections [1,2]. Flavonoids are secondary products of plants and are thus ingested daily from fruits, vegetables and other foods. Studies of dietary flavonoids have revealed a broad spectrum of biological activities for these molecules, including the inhibition of cell proliferation in cell culture, induction of apoptosis, alterations in the activity of certain intracellular enzymes, and antioxidant properties [3]. Most importantly, flavonoids show almost no toxic effects on normal peripheral blood and myeloid cells, or on normal hepatocytes [4,5]. Baicalein, a bioactive flavonoid extracted from the roots of *Scutellariabaicalensis* or *Scutellaria radix*, has been shown to exert antitumor activity [6]. In addition, this compound causes cell cycle arrest and suppresses the proliferation of cancer cells. Moreover, baicalein induces apoptosis in a variety of human cancer cell lines [7,8,9]. Moreover, baicalein inhibits cell migration and invasion through inhibiting MMP-2/9 activity in human hepatoma cells and human breast cancer cells [10]. In human skin carcinoma, baicalein inhibits cell invasion through inhibiting an anchor protein Ezrin expression [11]. Recently, baicalein is proven to be genotoxic without producing chromosomal alterations and mutagenesis, which results in the severe side effect in cancer chemotherapy [12]. According to the above data, baicalein is a candidate worth development in anticancer therapy.

Breast cancer is the most frequently diagnosed cancer in women worldwide, and according to the data from the World Health Organization, it comprises 16% of all female cancers. Owing to the advances in early diagnosis, improved surgical techniques, adjuvant therapies and the advent of various targeted therapeutic approaches, mortality from breast cancer has been progressively declining over the past two decades. A major challenge currently facing the scientific community is that despite these advances, a large number of breast cancer patients present with metastatic cancer or relapse and metastasize after initial response to standard of care therapy. It has previously identified an angiogenic regulator, Cyr61, as a protein potentially involved in breast cancer progression and metastasis [13].CYR61 is a heparin-binding, extracellular matrix-associated protein that belong to the CCN protein family, and is implicated in diverse biological processes such as cell adhesion, proliferation, differentiation, and survival via its multivalent affinity to several factors including cell surface proteins, matrix proteins and extracellular cytokines [14,15]. Intriguingly, CYR61 is also defined as a member of the matricellular protein group [16]. Secreted matricellular protein enables the modulation of cellular interactions with the extracellular matrix by its binding to structural matrix proteins, such as collagen, and its abrogation of focal adhesions, conferring a counter-adhesive effect on cells [17]. In cancer, over-expression of CYR61 was found in several cancers and resulted in tumor cell proliferation and EMT [18]. Epithelial-mesenchymal transition (EMT) is an essential process in multiple biochemical changes, including embryonic development, tissue remodeling, and wound healing. Recently, EMT has been shown to play a crucial role in tumor invasion and metastasis

[19]. EMT is a reversible phenotypic conversion that often occurs at the invasive front of many metastatic cancers [20]. In the process of EMT, epithelial cells reduce intercellular adhesion, acquire mesenchymal characteristics and increase invasive and migratory properties. Several transcriptional factors have been implicated in the control of EMT, including Snail, Slug, ZEB1, and Twist [21]. The zinc-finger transcription factor Snail and Slug has been characterized as a key EMT regulator [22]. Expression of Snail and Slug triggers EMT in breast cancer cells by repressing E-cadherin expression [23]. Therefore, the loss of E-cadherin expression was considered as a well-defined feature of EMT. CYR61 play a critical role for the induction of EMT-related genes that promote invasion and metastasis in gastric cancer[24], chondrosarcoma[25] and pancreatic ductal adenocarcinoma [26]. Therefore, searching for the phytochemicals that attenuate cancer invasion and metastasis by targeting CYR61, are very important for cancer treatment. Here we showed the first time that Baicalein suppresses the EMT by modulating CYR61 expression and its downstream signaling pathway.

Recently, Snail and Slug were found to be dually regulated by GSK3β through protein stability and cellular localization. Glycogen synthase kinase 3β (GSK3β) is a ubiquitously expressed serine/threonine kinase that is active in resting epithelial cells [27]. GSK3β can regulate Snail and Slug function via phosphorylation at two consensus motifs: phosphorylation at the first motif regulates their ubiquitination by β-transducin repeat containing protein (β-TrCP), whereas phosphorylation at the second motif controls their subcellular localization [28]. A non-phosphorylated variant of Snail and Slug residues more stably in the nucleus to exclusively induce EMT [11]. GSK3β can

inhibit Snail and Slug expression via inhibiting their transcription [20], as well as by regulating degradation and nuclear translocation [23]. Therefore, GSK3β maintain epithelial phenotypes via inhibiting the expression and stabilization of Snail and Slug and thereby also helps in maintaining a high E-cadherin expression [29]. Recently, growing evidences indicate that the analysis of Snail protein and its associated functional regulation is now on the "top of the wave" and is emerging as an important mechanism for the control of EMT. LOXL-2 is a member of the lysyl oxidase (LOX) gene family, which contains prototypic LOX and LOX-like proteins 1 through 4 (LOXL-1, LOXL-2, LOXL-3, LOXL-4) [25,26]. LOX is a copper-containing enzyme that catalyzes the oxidative deamination of the γ-amino group in certain peptydyl lysine residues and the resulting aldehyde groups spontaneously condense with vicinal peptydyl aldehydes or γ-amino groups of lysine to form covalent cross-linkages [30,31]. Although the catalytic mechanism of LOX-like enzymes has not been studied so far it is assumed that it will be similar to that of prototypic LOX enzyme [32]. Snail was found to interact physicallyand functionally with LOXL-2 [33]. The functional collaboration of LOXL-2 with Snail to repress E-cadherin expression is strictly dependent of the presence in Snail protein of two specific lysine residues: K98 and K137 which are critical for Snail stability [33], GSK3β interaction and protein ubiquitylation [23]. Based on these finding a hypothetical model is emerging in which LOXL-2 is envisioned as counteracting the action of GSK3β on Snail.

Given that EMT plays an integral role in sustaining the metastatic progression of breast cancer, developing more-effective, non-endocrine, non-toxic therapeutic strategies to

target EMT is highly desirable. In the present study, we specifically investigated the potential of baicalein to inhibit EMT, an early stage in cancer metastasis and examine the underlying molecular mechanisms in human breast cancer MDA-MB231 cell. We provide strong evidence that baicalein inhibits EMT in MDA-MB231 cell by modulating the mesenchymal and epithelial marker profiles. Baicalein targeted CYR61 to inhibit the activation of Akt, subsequently enhance activity of GSK3β which stimulate the ubiquitylation of Snail and Slug by direct association. Moreover, stabilization of Snail and Slug induced by interaction with LOXL-2 were attenuated by baicalein. Our data potentiate baicalein as an alternative regimen for eliminating metastasis in breast cancer patients.

2. Materials and Methods

2.1 Cell culture and reagents

MDA-MB231 was cultured in DMEM medium and MCF-7 breast carcinoma cells (ATCC, Manassas, VA) was cultured in mixture of F-12K and DMEM medium. Cultured media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic. All cultures were maintained at 37 ºC in a humidified incubator with 5% CO_2.

RPMI 1640 medium, bovine serum albumin, trypsin/ ethylenediaminetetraacetic acid, fetal bovine serum (FBS), and Antibiotic–Antimycotic 100X were purchased from Invitrogen (USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Baicalein and anti-β-actin antibodieswere purchased from Sigma Chemical Co. (USA). Baicalein was dissolved in DMSO. Anti-Slug , -Vimentin, -E-cadherin, -GSK3β, - pGSK3β, - Akt, -pAkt, - mouse anti-rabbit IgG (conformation specific) 5127S which does not recognize IgG heavy (50 kDa) or light (25 kDa) chains were purchased from cell signaling (USA). Anti-Snail, -LOXL-2, -ubiquitin antibodies and MG132 were purchased from Santa Cruz Biotechnology (USA). Polyvinylidene fluoride membranes for Western blotting were purchased from Millipore (USA).

2.2 Cytotoxicity

Cells were plated in 96-well plates in 200 µl medium containing 10% heat-inactivated FBS. After treatment, 20 µl of MTT reagent (5 mg/ml) were added, and cells were incubated for 3–4 h. Supernatants were then removed from the well, and 150 µl of DMSO were added. All experiments were conducted in quadruplicate. Cell viability was determined from the absorbance at 570 nm, measured using a Sunrise microplate reader (Austria). Cell viability is shown as the percentage of control viability (mean ± SD).

2.3 Wound healing assay

The cells were cultured in 6-well plates and grown in medium containing 10% FBS to nearly confluent cell monolayer, then carefully scratched using a plastic pipette tip to draw a linear "wound" in the cell monolayer of each well. The monolayer was washed twice with PBS to remove debris or the detached cells from the monolayer, and then drugs were added. The cultures were incubated at 37°C and observed by microscope (Olympus, Essex, UK) after 24h. Under the microscope, the number of cells that migrated into the cell-free zone, based on the line of the linear "wound" was evaluated.

2.4 Invasion assay

Cells (7×10^4) in 100 µl medium (without FBS) treated with or without 25 µM baicalein for 24 h were seeded in the upper chamber. Then 600 µl medium with 10% FBS was added to the lower chamber and served as a chemotactic agent. After 24 h incubation, the cells in the upper chamber were fixed in 4% paraformaldehyde for 20 min. Then the Matrigel was mechanically removed from the filter with a cotton swab. The cells

adhering to the under-side of the filter were stained with crystal violet and counted under microscope.

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

Total RNA from cell lines were extracted with TRIzol reagent (Invitrogen). Reverse transcription was carried out using the reverse transcription system (Promega, USA). Polymerase chain reaction (PCR) primers for amplification were as follows: Snail, forward 5'-GAGGACAGTGGGAAAGGCTC-3', reverse 5'-TGGCTTCGGATGTGCATCTT-3'; Slug, forward 5'-GAACTCACACGGAGAAG-3', reverse 5'-ACACAGCAGCCAGATTCCTC-3'; Vimentin, forward 5'-AATGGCTCGTCACCTTCGTGAAT-3', reverse 5'-CAGATTAGTTTCCCTCAGGTTCAG-3'; E-cadherin, forward 5'-GGAAGTCAGTTCAGACTCCAGCC-3', reverse 5'-AGGCCTTTTGACTGTAATCACACC-3'; GAPDH, forward 5'-GAGAAGGCTGGGGCTCATTT-3', reverse 5'-AGTGATGGCATGGACTGTGG-3'. RT-PCR was performed using Taq polymerase (iNtRON Biotechnology Inc., Korea). PCR was initiated by incubating the samples at 95℃ for 5 min, followed by 35 cycles of 40 s denaturation at 95℃, 40 s annealing at 55.4℃ (for Snail), 57.5ºC (for Slug and GAPDH), 58ºC (for Vimentin), 58.7ºC (for E-cadherin) and 5 min elongation at 72℃. Samples were analyzed by electrophoresis on 1.2% agarose gels containing 0.002% nucleic acid staining solution (RedSafe^{TM}; Biotechnology Inc., Korea).

2.6 Western Blotting

The cells were seeded and then treated for 24h. Then, the cells were harvested and lysed in RIPA buffer and kept on ice for 30 min. BCA assay was carried out and equal amounts of protein samples were loaded per well. Aliquots of the lysates were separated on SDS-polyacrylamide gels and transferred to PVDF membranes using a glycine transfer buffer. After blocking with 5% non-fat fried milk, the membranes were incubated overnight with primary antibodies, and then for 1h with secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.5% Tween 20. All the primary antibodies (with the exception of the anti-β-actin antibody) were used at a dilution of 1:1000. The anti-β-actin primary and secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG) (Vector Laboratories, USA) were used at a dilution of 1:10,000. Protein bands were detected using the WEST-ZOL® plus Western Blot Detection System (iNtRON, Korea).

2.7 Immunoprecipitation

Treated cells were harvested, washed with cold PBS, and lysed in 500µl lysis buffer (20 mMTris [pH=7.5], 150 mMNaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin and 1x protein inhibitor cocktail). Lysates were incubated with primary antibodies with gentle rocking overnight at 4ºC. Protein A/G agarose beads (25 µl of 50% bead slurry) were then added and samples were incubated with gentle rocking at 4ºC for 3h. Samples were then centrifuged at 14,000 rpm for 1 min at 4ºC, and the pellets were

resuspended with 40 µl 2x SDS sample buffer and vortexed. They were then heated at 100ºC for 10 min, separated on SDS-PAGE gels (10-12%), and transferred to PVDF membranes using transfer buffer (0.2 mM glycine, 25 mMTris-HCl [pH=8.8], and 20% [v/v] methanol). Membranes were then probed by western blotting using the protocol recommended by Cell Signaling Technology.

2.8 Transient transfection

The plasmid expressing CYR61 and Empty vector were prepared. Cells were transfected with CYR61 and Empty vector using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and treated as indicated. 24h post-transfection, the cells were harvested and cell lysates were subjected to immunoblot analysis.

2.9 Statistical analysis

Group comparisons were performed using the SPSS v.12.0 software with one-way analysis of variance (ANOVA) and student's t-test. $P<0.05$ and $P<0.001$ were considered to indicate statistically significant differences. All experiments were performed in triplicate.

3. Results

3.1 MDA-MB231 is more metastatic than MCF-7

Recent evidence has suggested that the cells undergo EMT to detach from their primary epithelial sites to mesenchymal tissue to form secondary tumors [19]. During the invasion process, EMT is activated via the increased expression of mesenchymal markers and decreased expression of epithelial markers [20]. Consistent with this, we observed increased migratory activity (Fig. 1A) and elevated expression of mesenchymal markers, such as Vimentin, Snail and Slug, whereas the epithelial marker E-cadherin was not detected in MDA-MB231 cells compared with that of MCF-7 (Fig. 1B). Since CYR61 and LOXL-2 was differently expressed in breast cancer cells and resulted in tumor cell proliferation and migration [13,33], we checked the mRNA and protein expression level of CYR61 and LOXL-2 in MDA-MB231 and MCF-7. The results showed that CYR61 and LOXL-2 were over-expressed in MDA-MB231 (Fig. 1C), suggesting that human breast cancer MDA-MB231 cell is more metastasis than MCF-7 cell.

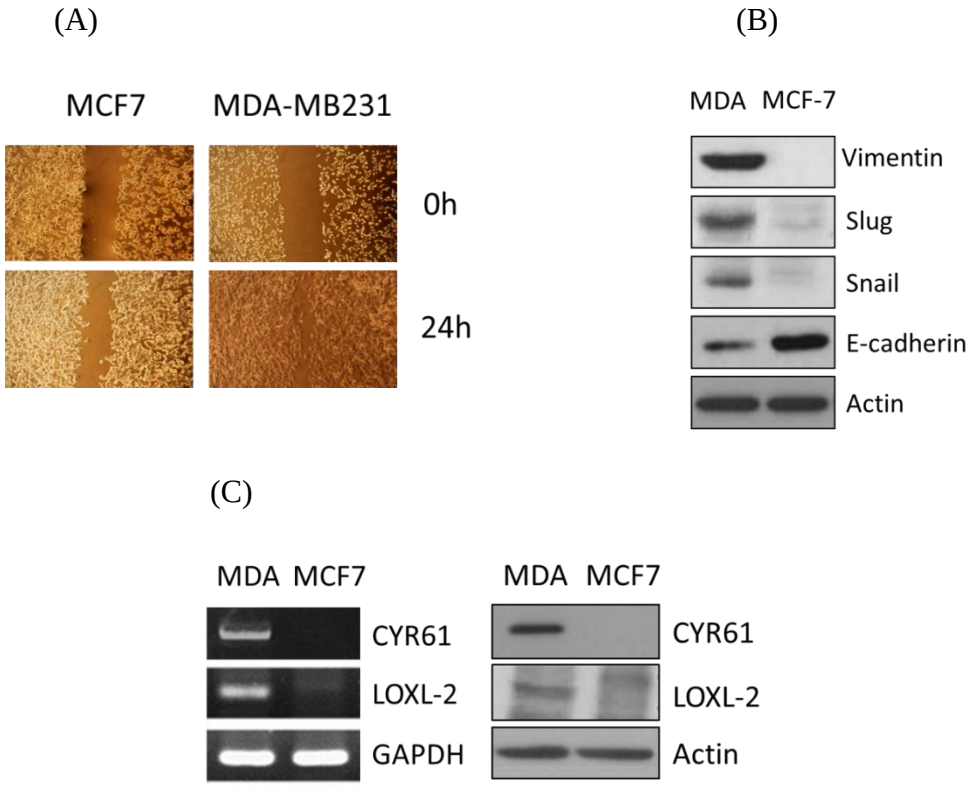


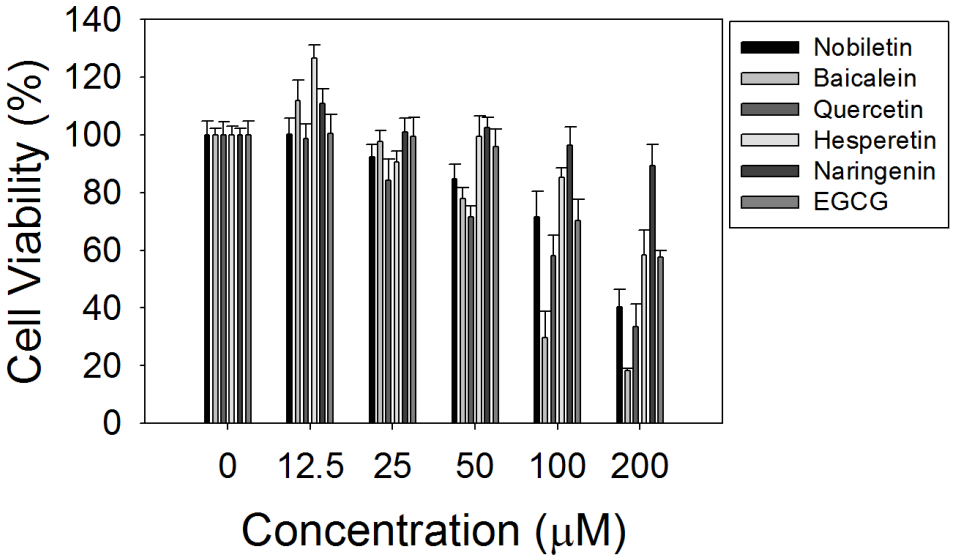
Figure 1.MDA-MB231 is more metastatic than MCF-7. (A) For the wound healing assay, 90% confluent cultures MDA-MB231 and MCF-7 were scraped to form a wound , and then incubate in 37 ºC with 5% CO_2 for 24 h. Photos of the wound were captured by microscopy. Relative distance of the wound width was measured and divided by the initial half-width of the wound. (B) Protein expression of mesenchymal and epithelial markers in MDA-MB231 and MCF-7. Cellular proteins of MDA-MB231 and MCF-7 were separated by SDS-PAGE and probed with appropriate primary antibodies. β-actin was used as internal control. (C) mRNA and protein level of CYR61 and LOXL-2 in MDA-MB231 and MCF-7 were determined by RT-PCR and western blotting, respectively.

3.2 Effect of Phytochemicals on MDA-MB231 human breast cancer cells

To eliminate the possibility that the suppression of migration by phytochemicals is due to cytotoxicity, cell viability was determined by MTT assay. Fig. 2A shows that a 24h treatment of MDA-MB231 with 6 phytochemicals at low concentrations (nobiletin 0-25 μM, baicalein 0-25 μM, quercetin 0-12.5 μM, hesperetin 0-50 μM, naringenin 0-100 μM, EGCG 0-50 μM) has no significant effect on cell survival despite the fact that a dose-response inhibition of cell growth was observed at high concentrations. These low concentration ranges were therefore applied in all subsequent experiments.Cancer cells undergoing EMT have been considered as an early event that leads to local invasiveness and distant metastasis. We initially performed a scratch wound healing assay. This procedure enables the assessment of whether phytochemicals affect cell motility. Fig. 2B illustrates the wounds treated with phytochemicals in 24 h in which baicalein, quercetin and hesperetin exhibited significantly dose-related delays in healing of MDA-MB231.As shown in Fig. 1, MDA-MB231 is more metastasis than MCF-7 and has high expression of CYR61 comparing with that of MCF-7. It has been reported that CYR61 plays important role in breast cancer progression and aggressiveness. We therefore examined the expression of CYR61 with 3 phytochemicals (baicalein, quercetin and hesperetin) that inhibited cell mobility to figure out which phytochemical could target CYR61 for its anti-metastatic activity. The results showed that only baicalein attenuatedthe expression of CYR61 (Fig. 3). In order to confirm the anti-metastatic effect of baicalein, invasion assay was performed (Fig. 4). After 24 h incubation,

baicaleinsuppressed cell invasion of human breast cancer cell MDA-MB231 with a dose-dependent manner. Collectively, baicalein can be a potential anti-metastasis compound by targeting CYR61.

(A)


Cell Viability (%)
140
120
100
80
60
40
20
0
0
12.5
25
50
100
200
Concentration (μM)
Nobiletin
Baicalein
Quercetin
Hesperetin
Naringenin
EGCG

(B)

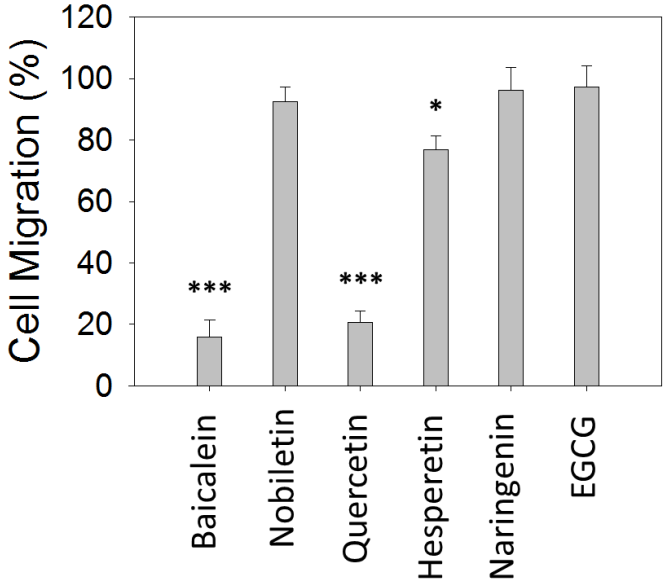

Cell Migration (%)
120
100
80
60
40
20
0
***
***
*
Baicalein
Nobiletin
Quercetin
Hesperetin
Naringenin
EGCG

Figure 2.Phytochemical effect on human breast cancer cell MDA-MB231. (A) Cells were seeded, incubated with indicated concentrations of nobiletin, baicalein, quercetin, hesperetin, naringenin and EGCG for 24 h. Cell viability was determined on the basis of MTT reduction. Values are the mean ± SD of three independent experiments. (B) Cells were treated with or without nobiletin 25 µM, Baicalein 25 µM, quercetin 12.5 µM, hesperetin 50 µM, naringenin 100 µM and EGCG 50µM for 24 h, then cells were used to perform wound healing scratch assay for additional 24 h. The wound areas were observed by microscope.

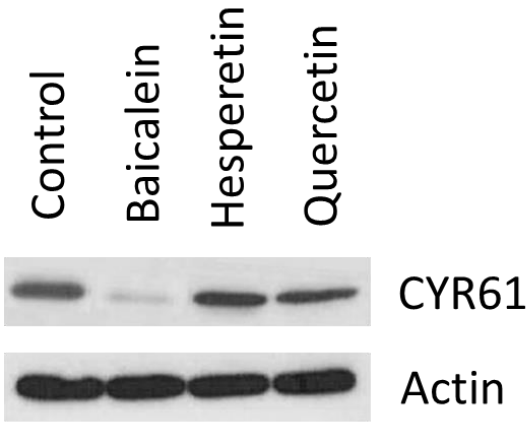


Figure 3.Phytochemical effect on CYR61 expression of human breast cancer cell MDA-MB231. The cells were seeded and treated with or without baicalein 25 µM, Hesperetin 50 µM and Quercetin 12.5 µM. Then, the cells were harvested and cellular proteins were separated by SDS-PAGE and probed with appropriate primary antibodies. β-actin was used as internal control.

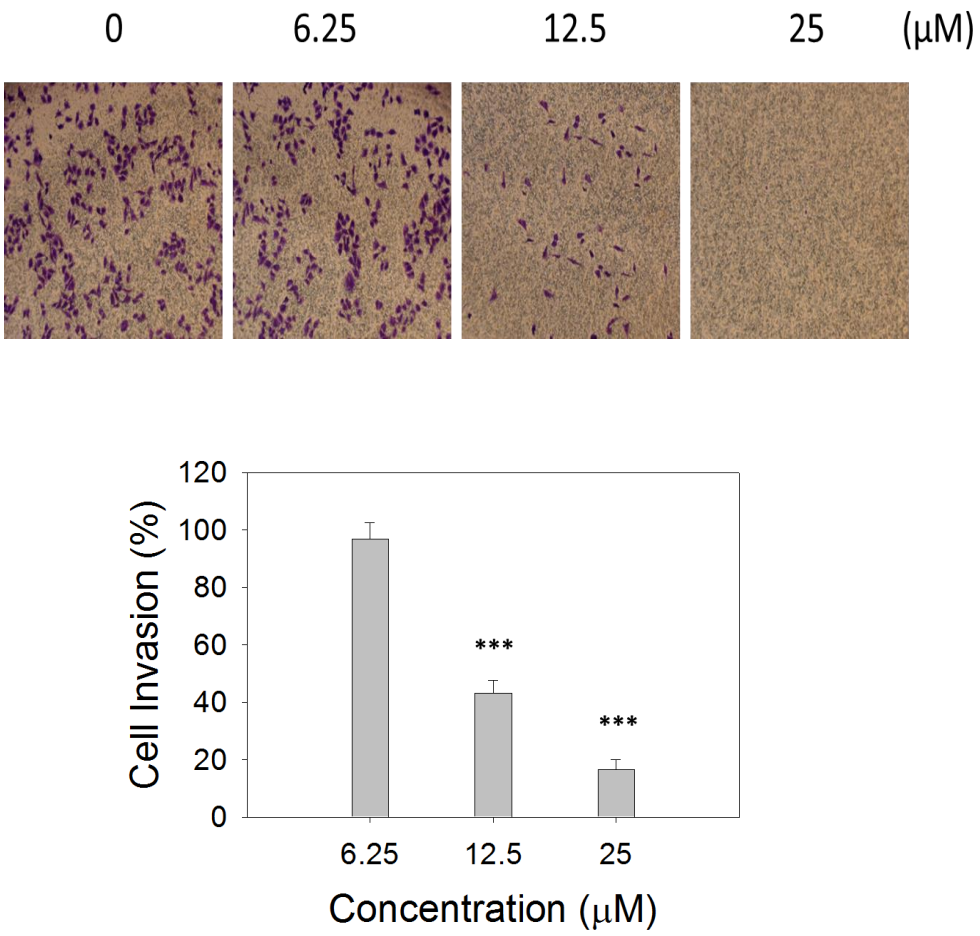


Figure 4. Baicalein reduces invasion ability of MDA-MB231. The cells were cultured in matrigel-invasion chambers followed by treatment with indicated concentrations of baicalein for 24 h. The number of cells that invaded through the matrigel was counted in five different regions. ****P<0.001*, compared with untreated control.

3.3 Baicalein suppresses migration of MDA-MB231 by post-transcriptional regulation of Snail and Slug

To ascertain whether the reverse effect of baicalein on EMT-related proteins in breast cancer MDA-MB231 cell, the exposure of MDA-MB231 cells to various concentrations (0 μM to 25 μM) of baicalein resulted in a dose-dependent increase in E-cadherin expression, along with a gradual decrease in Snail, Slug, CYR61 and LOXL-2 expression (Fig. 5A). Interestingly, baicalein significantly decreased Snail and Slug protein levels, but not mRNA level (Fig. 5B). However, baicalein treatment altered neither mRNA nor protein levels of E-cadherin, CYR61 and LOXL-2. This result suggested that down-regulation of Snail and Slug by baicalein was occurring at the post-transcriptional level.Moreover, baicalein did not show any effect to wound healing and EMT-related gene of MCF-7 (Fig. 6), suggesting that EMT regulation of baicalein is specific for MDA-MB231.

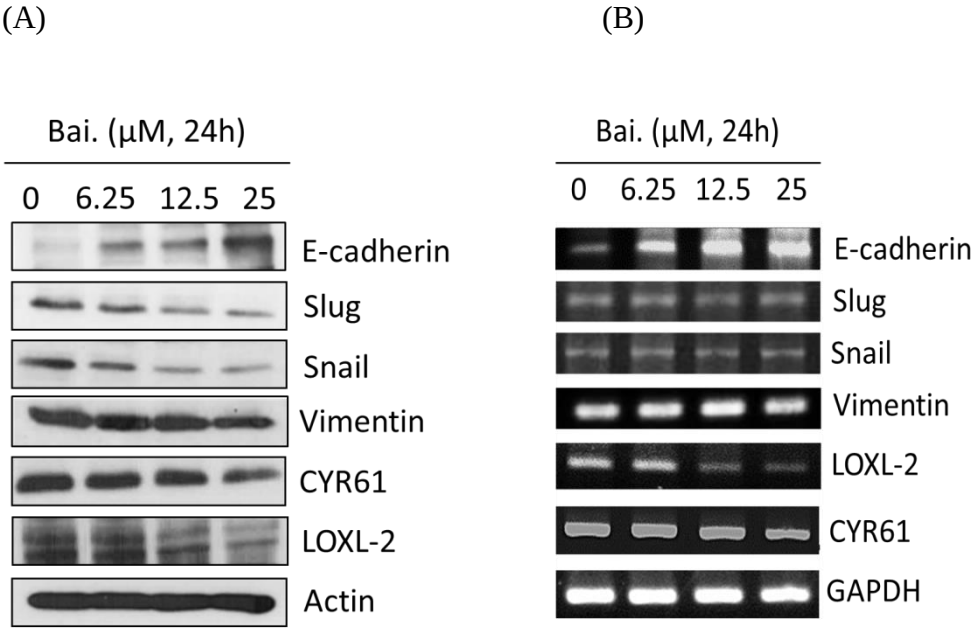
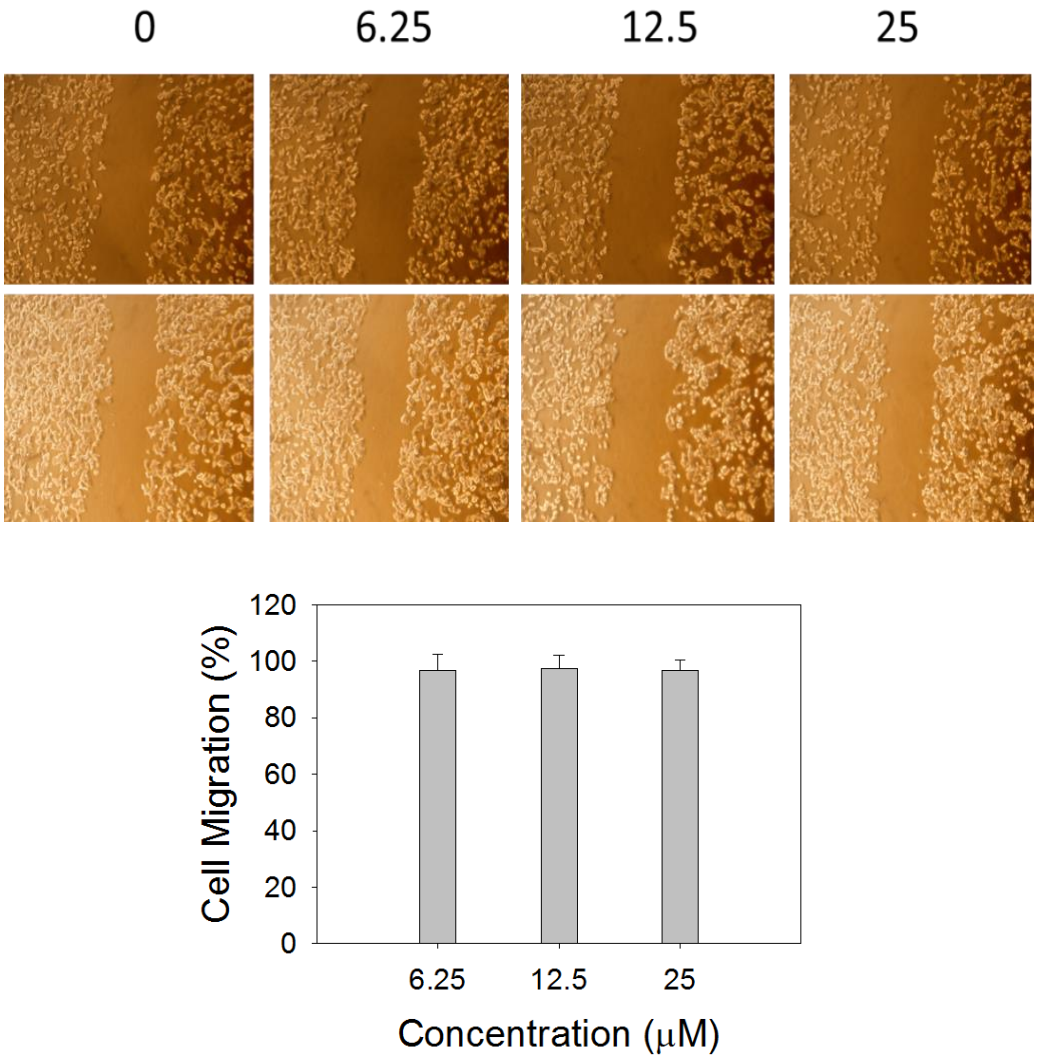


Figure 5. Baicalein induces post-transcriptional regulation of Snail and Slug in MDA-MB231. (A) MDA-MB231 cells were treated with or without baicalein for 24 h, and the expression of EMT markers, CYR61 and LOXL-2 were analyzed by western blotting. β-actin serves as the loading control. (B) MDA-MB231 cells were treated with or without baicalein for 24 h, and the mRNA levels of EMT markers, CYR61 and LOXL-2 were analyzed by RT-PCR. GAPDH serves as the loading control.

(A)


0
6.25
12.5
25
Cell Migration (%)
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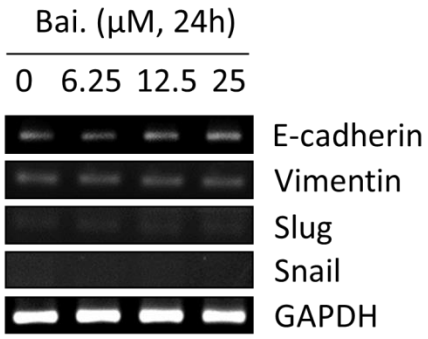
100
80
60
40
20
0
6.25
12.5
25
Concentration (μM)

(B)


Bai. (μM, 24h)
0 6.25 12.5 25
E-cadherin
Vimentin
Slug
Snail
GAPDH

(C)

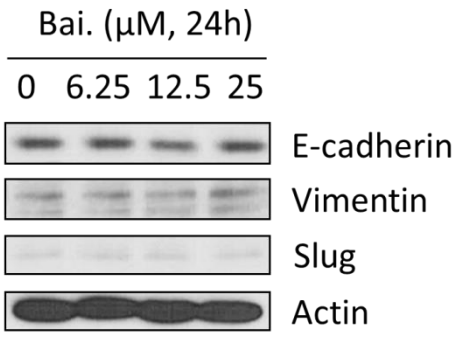

Bai. (μM, 24h)
0 6.25 12.5 25
E-cadherin
Vimentin
Slug
Actin

Figure 6.Baicalein effects on MCF-7. (A) MCF-7 were seeded and scraped to form a wound . Then, the cells were treated with or without baicalein with indicated concentrations for 24 h. Photos of the wound were captured by microscopy. Relative distance of the wound width was measured and divided by the initial half-width of the wound. (B) The cells were seeded and treated with or without baicalein for 24 h. mRNA expressions of mesenchymal and epithelial markers in MCF-7 were examined by RT-PCR. GAPDH served as an internal control. (C) Cellular proteins of MCF-7 were separated by SDS-PAGE and probed with appropriate primary antibodies. β-actinwas used as internal control.

3.4 Baicalein induced ubiquitylation of Snail and Slug by inhibiting the association of Snail and Slug with LOXL-2

Because the protein stability of Snail and Slug are regulated via ubiquitin-mediated proteasomal degradation processes, we speculated whether the degradation of Snail and Slug by baicaleinare mediated by induction of Snail and Slug ubiquitylation. To test this hypothesis, MDA-MB231 cells were treated with baicalein or the proteasomal inhibitor MG132 for 6 h, and then Snail and Slug were immunoprecipitated. The ubiquitylation state of Snail and Slug were detected by western blotting with an anti-ubiquitin antibody. The results revealed that compared with baicalein, MG132 treatment alone or cotreatment of MG132 and baicalein dramatically induced the expression of Snail and Slug (Fig. 7). As shown in Fig. 8, the ubiquitylation of Snail and Slug were increased by baicalein treatment compared with control. Based on these observations, we assessed that Baicalein down-regulated Snail and Slug via proteasomal degradation and ubiquitylation. Since LOXL-2 has been known as potential Snail's partner for Snail stabilization and collaborating in EMT, therefore, we checked whether baicalein affects to the association of LOXL-2 with Snail and Slug. As expected, treatment of baicalein reduced the binding of LOXL-2 with Snail (Fig. 9A). Interestingly, we found that Slug can also be bound with LOXL-2 and the interaction can be inhibited by baicalein (Fig. 9B). The previous data showed the inhibition of LOXL-2 expression by baicalein, suggesting that the ability of baicalein to reduce the interaction of LOXL-2 with Snail and Slug might simply be because of the down-regulation of LOXL-2 expression.

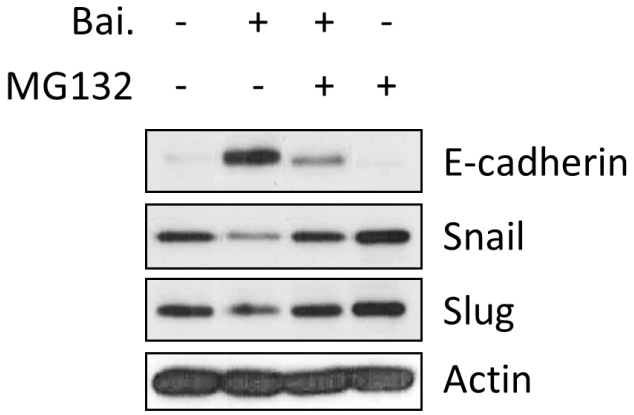
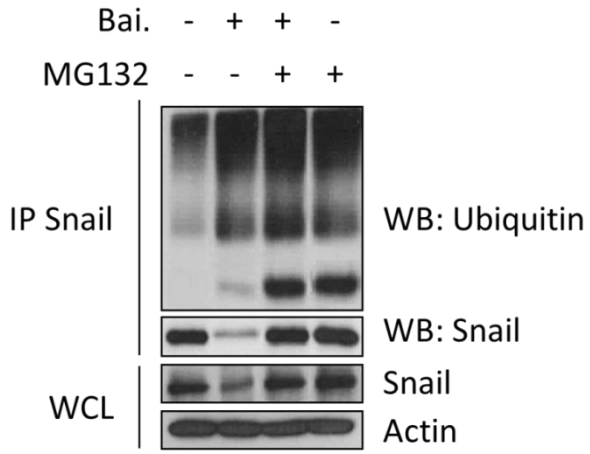


Figure 7.Baicalein regulates stability of Snail and Slug via ubiquitin-mediated proteasomal degradation processes. MDA-MB231 were treated with MG132 10μM for 6 h or cotreatment with baicalein 25 μM. Subsequently, western blot analysis was performed using E-cadherin, Snail and Slug antibodies. β-actin served as loading control.

(A)



(B)

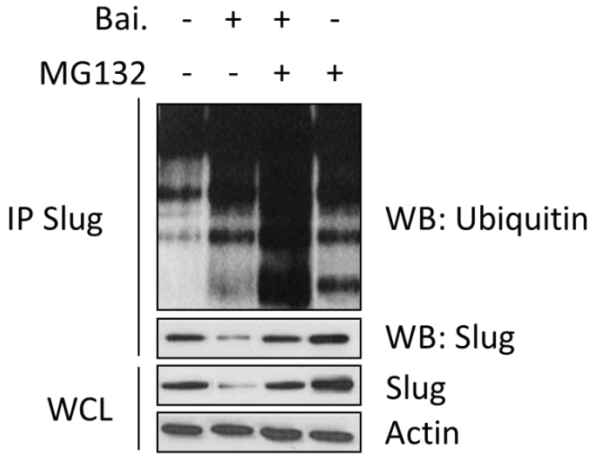
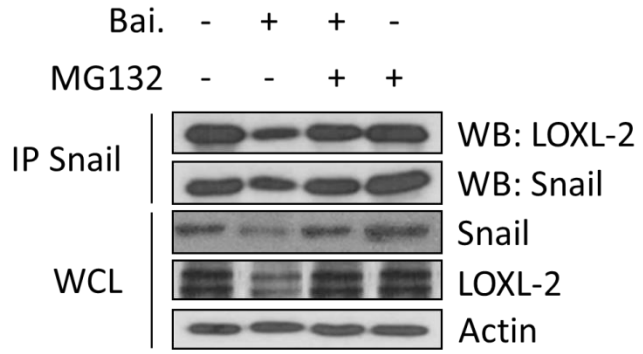


Figure 8.Baicalein induces ubiquitylation of Snail and Slug. MDA-MB231 were treated with MG132 10 μM for 6 h or cotreatment with baicalein 25 μM. Subsequently, the cells were harvested. After Snail and Slug were immunoprecipitated, the ubiquitylation of Snail (A) and Slug (B) were examined by western blotting. Western blot analysis was performed using E-cadherin, Snail and Slug antibodies. β-actin served as loading control.

(A)



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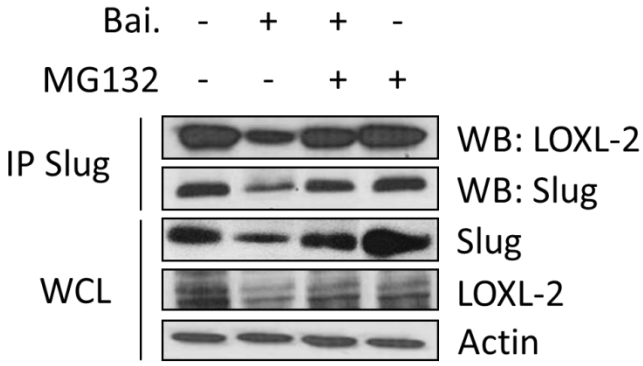


Figure 9.Baicalein reduces the binding of LOXL-2 with Snail and Slug. . MDA-MB231 were treated with MG132 10 μM for 6 h or cotreatment with baicalein 25 μM. Subsequently, the cells were harvested. After Snail and Slug were immunoprecipitated, the binding of Snail (A) and Slug (B) with LOXL-2 were examined by western blotting. Western blot analysis was performed using Snail, Slug and LOXL-2 antibodies. β-actin served as loading control.

3.5 Baicalein mediates Snail and Slug degradation via Akt/GSK3β signaling pathway

An increase in the free cytoplasmic pool of Snail and Slug is necessary, albeit insufficient, for EMT, unless GSK3β is inhibited[34]. To determine how baicalein affects the expression of Snail and Slug, as well as the subsequent conversion of EMT phenotypes, the role of GSK3β in a baicalein-triggered EMT reversal was explored. As shown in Fig. 10, baicalein markedly decreased the levels of GSK3β phosphorylation. Similarly, the phosphorylated status of Akt, an upstream kinase directly responsible for the phosphorylation and subsequent inhibition of GSK3β [35], was blocked by baicalein in a dose-dependent manner.

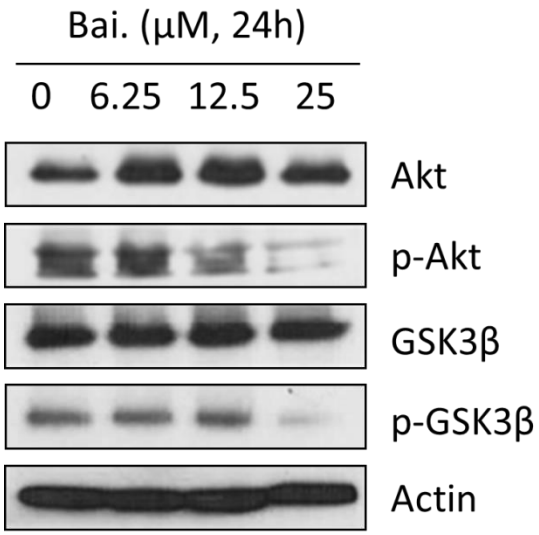


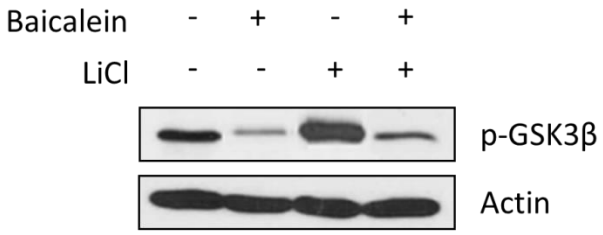
Figure 10.Upstream role of Akt/GSK3β signaling pathway in Baicalein-mediated downregulation of Snail and Slug stability.MDA-MB231 cells were treated with various concentrations of baicalein in 24 h. Subsequently, Western blot analysis was performed using specific antibodies against phospho-GSK3β (Ser9), GSK3β, phospho-Akt (Ser473) andAkt.

3.6 Baicalein induced ubiquitylation of Snail and Slug by stimulate the interaction of GSK3β with Snail and Slug

Since GSK3β is the main kinase that phosphorylates Snail and Slug and then induces the protein degradation of Snail and Slug, we next examined the role of GSK3β in the protein degradation of Snail and Slug under the treatment of baicalein. In order to test this hypothesis, the cells were treated with LiCl, a potent GSK3β inhibitor [36], in accordance with baicalein treatment, the expression of p-GSK3β was increased (Fig. 11A). Comparing with baicalein treatment, the cotreatment with baicalein rescued Snail and Slug and display EMT phenotype by the loss of E-cadherin expression (Fig 11B). Moreover, LiCl could stop the ubiquitylation of Snail and Slug that were induced by 24h incubation with baicalein and MG132 (Fig. 12). These results suggested that GSK3β plays important role in the ubiquitylation of Snail and Slug by baicalein treatment. The protein stability of Snail and Slug are regulated by LOXL-2 and GSK3β. LOXL-2 can bind with Snail at the same position in which GSK3β interact with Snail, it is suggested that LOXL-2 seems to attenuate the GSK3β-dependent Snail degradation. Therefore, we tested whether baicalein can alter the interaction of Snail and Slug with LOXL-2 that counteracts GSK3β action. As shown in Fig. 13, baicalein induced the binding of GSK3β with Snail and Slug, whereas association between LOXL-2 with Snail and Slug were inhibited. As expected, the inhibition of GSK3β induced by LiCl treatment stimulated the binding of Snail and Slug with LOXL-2. These results provided the direct evidences for the confrontative relationship between LOXL-2 and GSK3β in Snail and

Slug regulation. Taken together, baicalein stimulated the ubiquitylation of Snail and Slug by enhanced the binding of GSK3β with Snail and Slug.

(A)



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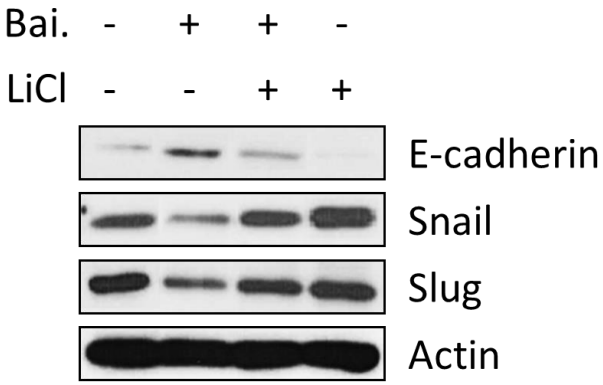
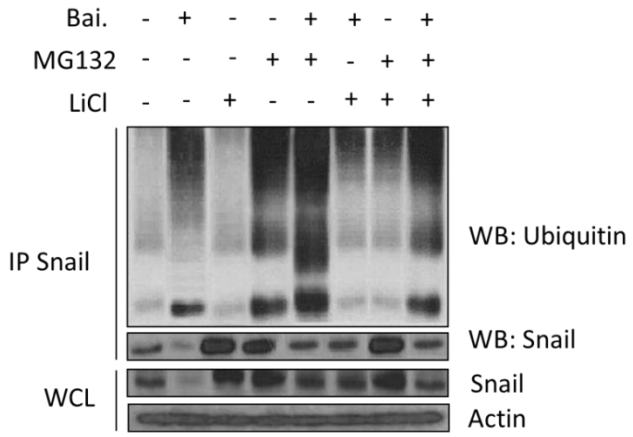


Figure 11.The role of GSK3β in Baicalein-mediated ubiquitylation of Snail and Slug. MDA-MB231 cells were treated with Baicalein (25 μM) or LiCl (50 mM) in 6 h or cotreatment of baicalein and LiCl. The expression of p-GSK3β (A) and EMT-related genes (B) was examined by western blotting.

(A)



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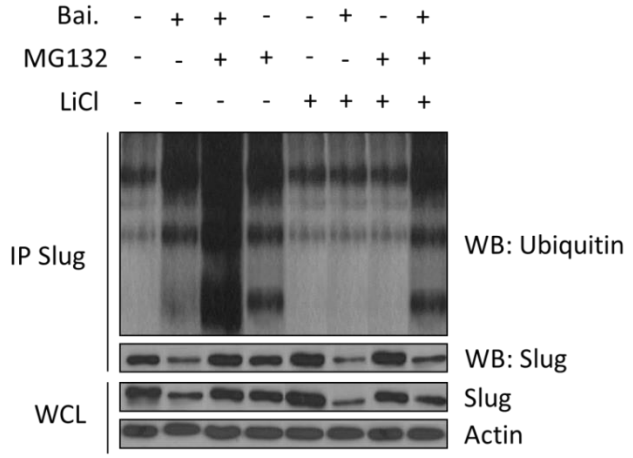
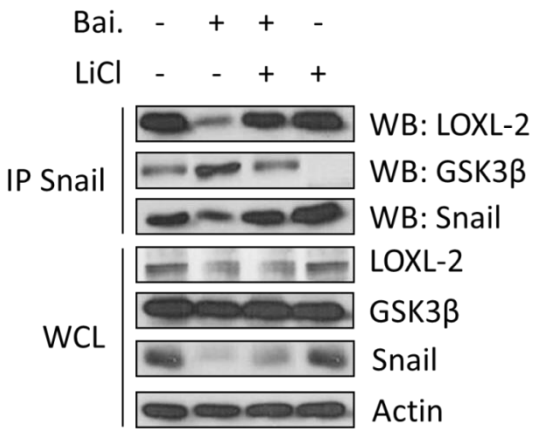


Figure 12. GSK3β inhibitor can inhibit the ubiquitylation of Snail and Slug. MDA-MB231 were treated with MG132 10 μM for 6 h or LiCl 50 mM for 6 h or cotreatment with baicalein 25 μM. Subsequently, the cells were harvested. After Snail and Slug were immunoprecipitated, the ubiquitylation of Snail (A) and Slug (B) were examined by western blotting. Western blot analysis was performed using E-cadherin, Snail and Slug antibodies. β-actin served as loading control.

(A)



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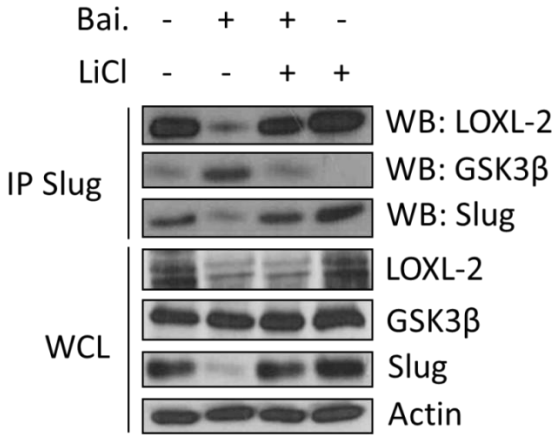

Figure 13.Baicalein stimulates binding of GSK3β with Snail and Slug and inhibits binding of LOXL-2 with Snail and Slug. MDA-MB231 cells were treated with baicalein (25 μM) or LiCl (50 mM) in 6 h or cotreatment of baicalein and LiCl. Then, Snail and Slug were immunoprecipitated. The binding of GSK3β and LOXL-2 with Snail (A) and Slug (B) were examined by western blotting.

3.7 CYR61 regulates Baicalein-mediated Akt/GSK3β signaling in human breast cancer MDA-MB231 cells.

According to previous data, baicalein inhibited cell migration of MDA-MB231, which contains over-expression of CYR61, but not MCF-7. It has been reported that CYR61 contributes to activation of Akt and the phosphorylation of Akt target GSK3β. To evaluate the mechanism underlying the effects by which baicalein targets CYR61-induced Akt activation and inhibition of GSK3β activity, transient transfected MCF-7 cells with CYR61 was carried out. Expression of CYR61 in MCF7-empty vector and CYR61 transfected MCF-7 were detected by western blotting (Fig. 14A). The results showed that CYR61-transfected MCF7 cell contains high expression of p-Akt and p-GSK3β which are down-regulated by baicalein (Fig. 8B). Moreover, over-expression of CYR61 induced EMT as evidenced by the loss of E-cadherin and induction of Snail and Slug. Consistent with previous data, 24 h baicalein incubation can cause EMT reversal in transfected cell (Fig. 14B).These findings demonstrated that baicalein inhibited CYR61-induced EMT via Akt/GSK3β pathway. Interestingly, transient transfection CYR61 in MCF7 cells could induce the expression of LOXL-2 which can be inhibited by baicalein treatment (Fig. 14C), suggested that CYR61 is upstream of LOXL-2. However, further study will be needed to investigate which molecular mechanism involves in CYR61 – LOXL-2 signaling pathway.

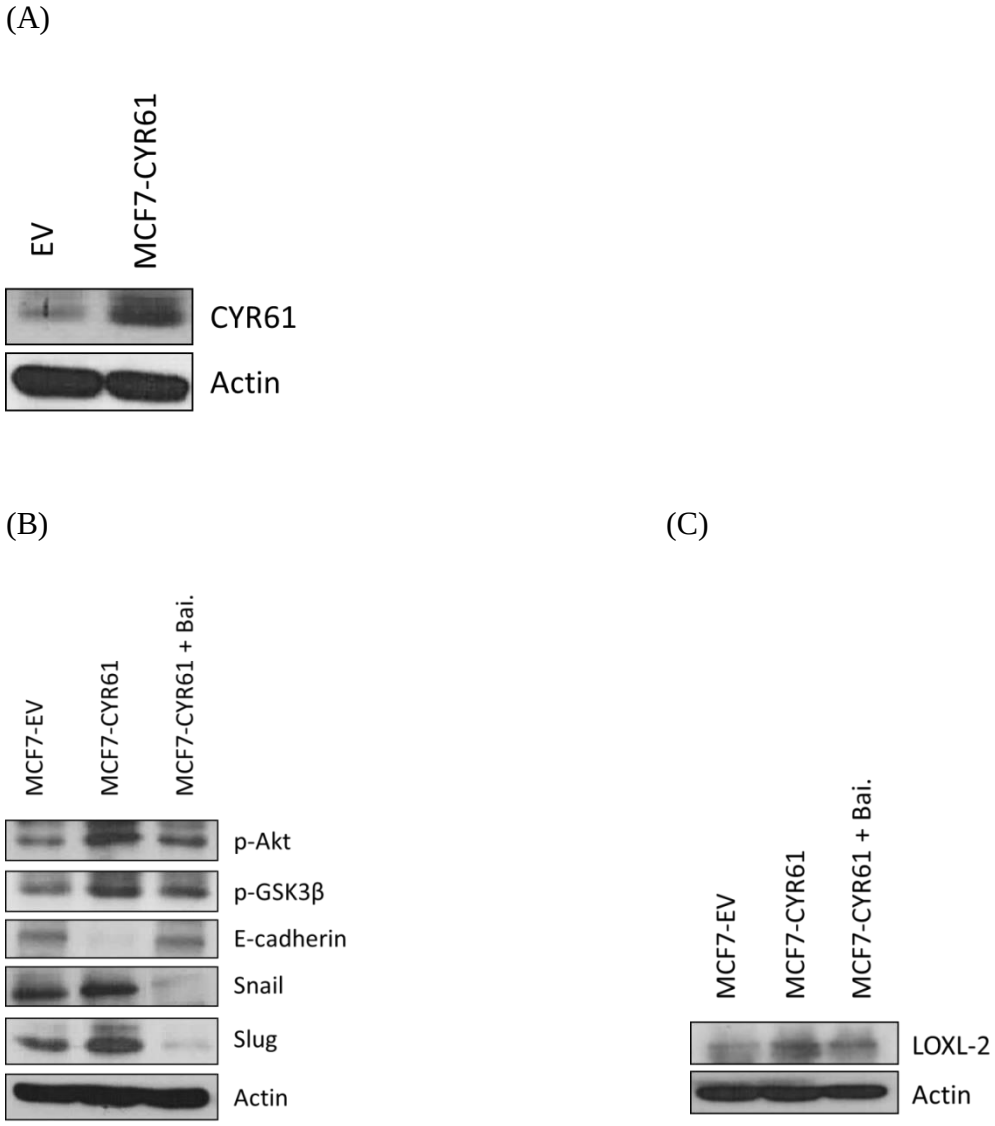

Figure 14.CYR61 plays an important role in Baicalein-mediated Akt/GSK3β signaling pathway. (A) Empty vector (control) or CYR61-expressing pcDNA3.0 were introduced into MCF-7 cells. Cellular levels of CYR61 were analyzed by Western blotting using β-actin as a loading control. (B)and (C) After 24 h of recovery, the cells were treated with or without baicalein and further incubated for an additional 24 h. Western Blot analysis was performed using indicated antibodies.

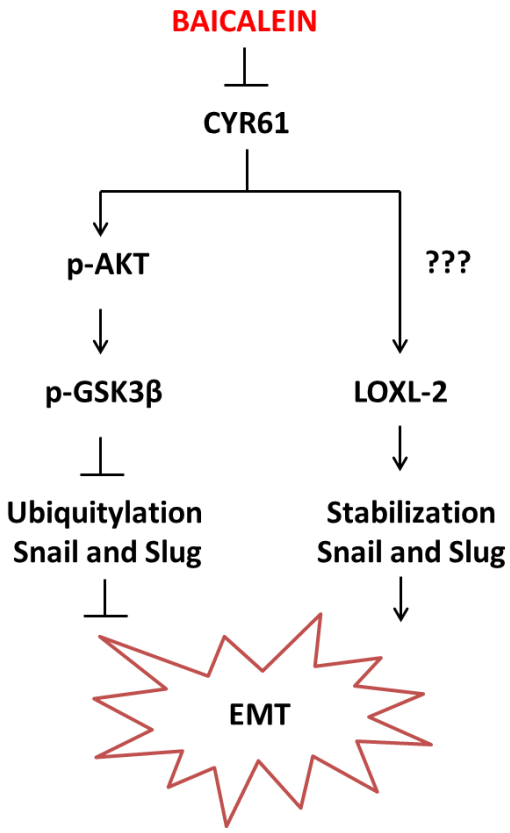

Figure 15.Suggested mechanism of Baicalein inhibit EMT in breast cancer cell MDA-MB231. Baicalein targeted CYR61 to inhibit the activation of AKT, subsequently enhance activity of GSK3β which stimulate the ubiquitylation of Snail and Slug by direct binding. Moreover, stabilization of Snail and Slug induced by interaction with LOXL-2 were attenuated by baicalein. By inhibited CYR61, baicalein also down-regulated LOXL-2 expression. However, molecular mechanisms show how CYR61 can regulate LOXL-2 need to be studied further.

4. Discussion

Breast cancer is the second most common cause of death and it is also known as the second leading cause of mortality. A half million women are dead each year by breast cancer. As metastatic breast cancer is the leading cause of cancer-related death among women in many countries, establishing and applying new treatments for breast cancer patients are important goals worldwide. baicalein is one of the major flavonoids in *Scutellariabaicalensis* that has long been widely used for thousands of years in oriental medicine. Several biological effects of baicalein such as anti-viral, anti-hepatotoxicity, anti-inflammation, and anti-tumor properties have been reported. However, the anti-metastatic effect and molecular mechanism of baicalein on breast cancer have less information.

Human breast cancer MDA-MB231 cell has higher expression of CYR61 and migration than that of MCF-7 (Fig. 1). Among phytochemicals that can affect to cell migration, only baicaleincan target CYR61 (Fig. 2) which has been implicated as an important mediator in proliferation and metastasis of breast cancer. The blockage of CYR61 might be a potent target for breast cancer treatment. It is worth to further investigate how baicalein regulates EMT by targeting CYR61.

EMT has been considered as the first step of tumor invasion and metastasis. Recent studies revealed that the expression profiles of EMT are correlated with tumor grades and metastasis of breast cancer [37]. Here we showed that baicalein can reverse EMT as evidenced by the increase of E-cadherin and the decrease of Snail and Slug (Fig. 5A).

Snail and Slug is a highly unstable protein with a short half-life and are regulated by a complex signaling network at both the transcriptional and post-transcriptional levels [38]. Interestingly, our data clearly showed that baicalein degrades Snail and Slug via post-transcriptional regulation processes (Fig. 5A and 5B). Recently, GSK3β has been characterized as kinase responsible for the phosphorylation of Snail and Slug [39]. Here we revealed baicalein was not only down-regulated p-GSK3β, it also inhibited activation of Akt, which is down-stream regulator to PI3K (Fig. 10). Akt is frequently activated in various cancers and plays a critical role in promoting EMT and invasion [40]. For instance, activation of Akt pathway is required for induction of TGFβ and EGF-dependent EMT [41]. Akt can also phosphorylate IKKα to increase Snail expression and induce EMT [42]. To date, GSK3β has been characterized as a main kinase responsible for the subcellular and protein stability of Snail [43]. Moreover, it has been reported that activation of upstream Akt signaling represses GSK3β activity. Marra et al. [44] and Vitale et al. [45] have reported that major role of CYR61 in the regulation of Akt-mediated pathway and in the sensitivity of cancer cells to anti-cancer drugs. Therefore, we hypothesize that baicalein can inhibit EMT via CYR61/Akt/GSK3β signaling pathway. As expected, overexpression of CYR61 induces Akt activation and inactive GSK3β and baicalein treatment attenuated this signaling pathway (Fig. 14B).

Interestingly, we found that CYR61 also can regulate LOXL-2 expression (Fig. 14C). However, the detailed mechanisms need to be studied further.Lysyl oxidase-like 2 (LOXL-2) is one of five members of the lysyl oxidase (LOX) family of extracellular matrix proteins and mediates the cross-linking of stromal collagens and elastin [46].

Other studies found LOXL-2 protein levels to be higher in poorly differentiated breast carcinomas, and elevated LOXL-2 mRNA was observed in invasive and metastatic breast cancer cell line [47].Furthermore, LOXL-2 has been shown to physically interact with Snail which might influence Snail activity [33]. Snail-LOXL-2 association depends on the SNAG domain and Snail's lysine residues K98 and K137 are critical for Snail stability [33].Interestingly, LOXL-2 seems to attenuate the GSK3β-dependent Snail degradation. Here we showed that baicalein inhibits EMT through attenuates binding of LOXL-2 with Snail and Slug (Fig. 9A and Fig. 9B), whereas stimulates binding of GSK3β with Snail and Slug (Fig. 13A and 13B) which can lead to the ubiquitylation of Snail and Slug (Fig.8A and Fig. 8B).

In addition to the activities of arresting cancer cell proliferation and inducing apoptosis, baicalein shows abilities to suppress metastasis, suggested by several reports, with the underlying mechanisms remain to be elucidated. Here we found a novel post-transcriptional mechanism that control Snail and Slug protein turnover in breast cancer MDA-MB231 cell by baicalein via targeting CYR61/Akt/GSK3β signaling pathway (Fig. 15). Moreover, stabilization of Snail and Slug by binding with LOXL-2 was inhibited under baicalein treatment (Fig. 15), suggesting that baicalein is potential candidate for anti-metastasis in breast cancer treatment.

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