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Investigation of CDK8 inhibitor Q-12 effects on CDK8 and CDK8 substrates in triple negative breast cancer cell line MDA-MB-468

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INVESTIGATION OF CDK8 INHIBITOR Q-12 EFFECTS ON CDK8 AND CDK8 SUBSTRATES IN TRIPLE NEGATIVE BREAST CANCER CELL LINE MDA-MB-468

By

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Investigation of CDK8 inhibitor Q-12 effects on CDK8 and CDK8 substrates in triple negative breast cancer cell line MDA-MB-468

Abstract

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Cyclin-dependent kinases (CDKs) and cyclins (Cyclins) are the core molecules in the regulation mechanism of the entire cell cycle. Cell cycle dysregulation is a common feature of human cancers, and inhibitors of cyclin-dependent kinases (CDKs) play a crucial role in cell cycle control and are one of the most promising areas of cancer therapy. We aspired to use our cyclin-dependent kinase 8 (CDK8) inhibitor, Q-12, as a probe for biomarker discovery for CDK8 inhibitor sensitive tumor types. Q-12 shows potent inhibition of cell viability and induction of apoptosis process in some triple-negative breast cancer (TNBC) and colorectal cancer cell lines in vitro. Western blot results indicate that Q-12 decrease p-STAT3 (Ser 727) stabilized p-STAT3 (Tyr 705) cause its upregulation. Cytokines are responsible for the increased phosphorylation of STAT3 (Tyr 705). Q-12 inhibit phosphorylation of CDK8 substrates STAT3 (Ser 727), STAT1 (Ser 727), E2F1 (Ser375) and reduce CDK8 protein levels. Q-12 initially increase E2F1 protein levels activated E2F1 and decrease Mcl-1 protein levels. All results suggest that STAT3 may not play a major role in cell death mechanism while E2F1 may play a major role. The main aim of the study is to investigate CDK8 inhibitor Q-12 effects on CDK8 and CDK8 substrates in triple negative breast cancer cell line MDA-MB-468, in order to better understand the mechanism of anti-proliferative effect of Q-12.

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APC	adenomatous polyposis coli
AKT	protein kinase B
ATP	adenosine triphosphate
CDK	cyclin-dependent kinase
CPT	cryptotanshinone
BCA	bicinchoninic acid assay
BH3	Bcl-2 homolog region 3
BSA	bovine serum albumin
Bcl-2	B-cell lymphoma 2
DMSO	dimethyl sulfoxide
ER	estrogen receptor
EGFR	epidermal growth factor receptor
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GI_{50}	half growth inhibition
HBSS	hank's balanced salt solution
HER2	human epidermal growth factor receptor 2
IAP	inhibitors of apoptosis proteins
IC_{50}	half maximal inhibitory concentration
IL-6	interleukin-6
NSCLC	non-small cell lung cancer
OSM	oncostatin M
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

STAT	signal transducer and activator of transcription
TAD	transactivation domain
TBST	tris buffered saline with Tween 20
TCF/LEF	T-cell factor/lymphoid enhancer factor
TNBC	triple negative breast cancer
TFIIH	transcription factor II H
PBS	phosphate buffered saline
PCD	programmed cell death
P-TEFb	positive transcription elongation factor b
PTEN	phosphatase and tensin homolog
Wnt	wingless-related integration site

Background

Cyclin Dependent Kinase

The cell cycle is the basic process of cell life activities, which controls the transition of cells from stationary phase to growth and proliferation phase. Cyclin-dependent kinases (CDKs) and cyclins (Cyclins) are the core molecules in the regulation mechanism of the entire cell cycle. Cell cycle dysregulation is a common feature of human cancers, and inhibitors of cyclin-dependent kinases (CDKs) play a crucial role in cell cycle control and are one of the most promising areas of cancer therapy. Cyclin-dependent kinase is one of the members of serine/threonine kinase family, which is a dimer complex composed of cell cycle catalytic kinase subunit and regulatory subunit, and 11 CDK members have been found so far. CDKs are present in multiple key regulatory pathways in all known eukaryotic cells and phosphorylate their substrates on serine and threonine (1). CDKs play important roles in controlling cell division and regulating transcription in response to a variety of extracellular and intracellular signals (2). CDK regulatory mechanisms rely on positive phosphorylation (CDK agonist kinase, CAK) and negative phosphorylation (Weel, Myt1), and related regulatory mechanisms with highly interconnected assembly of CDK-cyclin complexes that drive the cell cycle.

CDK family proteins regulate key checkpoints in the cell cycle and are well-validated targets for the treatment of various diseases. While major drug discovery has focused on classical ATP-competitive inhibitors, the emergence of new technologies has also driven research on non-classical CDK inhibitors, such as allosteric site inhibitors, covalent inhibitors, and non-ATP-competitive peptide mimics. MMD37K is the first non-ATP-competitive CDK4/6 inhibitor and is currently undergoing clinical studies. Its emergence will lead to the comparison of non-classical CDK inhibitors with existing ATP-competitive CDK inhibitors, promoting the development of a

new generation of CDK inhibitors. Palbociclib, abemaciclib and ribocicilib are all CDK4/6 selective inhibitors approved by the U.S. Food and Drug Administration (FDA) for the treatment of hormone receptor-positive metastatic breast cancer in combination with specific endocrine therapies (3). The successful development of these inhibitors is widely regarded as a breakthrough in the treatment of advanced breast cancer and will stimulate the development of cell cycle-targeted inhibitors.

In an evolutionary analysis of CDKs and cyclins in metazoans and their unicellular relatives, Cao., el (4) found that the CDK family can be divided into eight subfamilies. Seven subfamilies (CDK1/2/3, CDK5, CDK7, CDK 20, CDK8/19, CDK9, and CDK10/11) are conserved in metazoans and fungi, CDK4/6 subfamilies are found only in eumetazoans.

The success of CDK4/6 inhibitors lies in finding highly selective molecules with good pharmacokinetic properties. The development of other CDK subtype inhibitors in the future still needs to follow such rules. In addition, it is also very important to find tumor-sensitive and highly relevant CDK subtype targets for precise treatment rather than acting as cytotoxic drugs.

As for main function of cyclin-dependent kinase 8 in cancer, it responds to several intracellular signaling pathways, and it has been linked to many different processes, including cellular growth, proliferation and differentiation (5).

CDK8, a member of the mediator complex, is a 53 kD protein kinase containing 464 amino acids. It is active only when associated with its regulatory partner cyclin C (CycC). CDK8 is located at chromosome 13Q-12, whose amplification often exists in colorectal cancer (CRC) (6-8). Indeed, overexpression of CDK8 is observed in CRC along with gastric cancer and melanoma, etc. And CDK8 deletion exhibits antitumor activity (7,9-12).

CDK8 has multiple targets and phosphorylates several transcription factors, affecting their stability and activity. To date, the role of CDK8 in colorectal cancer has been shown to be the best in regulating β-catenin-dependent gene expression. Evidence suggests that CDK8 functions as a potent oncogene in colon cancer through the WNT/β-catenin signaling pathway co-activated in the p53 transcriptional program (13,14).

William Hahn et al. (15) found that CDK8 must be involved in the β-catenin-mediated proto-oncogene activation mechanism. However, Nicholas Dyson lab (16) found that the transcription factor E2F1 can down-regulate the activity of β-catenin, but this down-regulation can be inhibited by the tumor suppressor gene Rb and CDK8. It appears that CDK8 acts directly and indirectly to enhance β-catenin transcriptional activity and release it from E2F1 inhibition. CDK8 phosphorylates serine 375 in E2F1 both in vitro and in cells, and phosphorylation of this residue is required for the interaction of E2F1 with CDK8 and is dependent on CDK8 kinase activity. Phosphorylation of S375 by CDK8 regulates the ability of E2F1 to repress the transcription of β-catenin/TCF-dependent genes, as well as the activation of E2F1-dependent genes (17).

In the context of breast cancer CDK8 along with Skp2 has been shown in tissue samples to positively correlate with stage of breast cancer (18). In the context of ER+ breast cancer it has been shown that CDK8 inhibition suppressed estrogen induced gene transcription through reduced RNAPII phosphorylation suggesting a potential role of CDK8 inhibitors with antiestrogen therapy (19). Furthermore, miRNA mediated knockdown of CDK8 reduces proliferation and migration of breast cancer cells (20).

Through the above studies, we found that CDK8 is a promising new target, and small molecule intervention also provides a potentially very attractive target to precisely affect context-dependent gene expression (21). Since 2013, numerous CDK8-specific inhibitors have been

discovered and appear in patent applications. With greater understanding of CDK8 activity in cancer, we hope to develop robust response hypotheses and personalized clinical approaches (21,22).

Previous work done by Dr. Russu et al (23) has identified a novel kinase inhibitor known as Q-12, a novel compound that contains the direct attachment of the quinazoline fragment to the piperazinylpyrimideine scaffold (Figure 1), which has shown moderate selectivity toward CDK8 and c-KIT, another protein kinase involved in cellular signal transduction pathways.

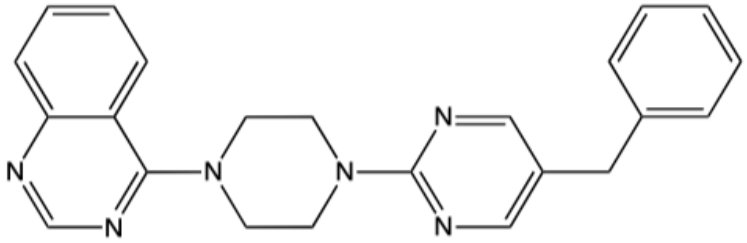

Figure 1. Chemical structure of Q-12

To test the potential of Q-12 to bind with the human kinome that was designed to target, a kinase binding assay was used to exam the ability of 10 μM of Q-12 to interfere with the binding of a given kinase with an immobilized, ATP directed agent. A single dose of 10 μM was chosen to allow for the detection of the kinases able to bind Q-12, followed by dose-response binding experiments. The competition with ATP for the binding and inhibition of CDK8 kinase and KIT kinases, is displayed as the dissociation binding constant (K_d) in Table 1.

Table 1
Kinase Binding and Inhibition Data for Q-12.

Kinase	K_d (μM)
CDK8	0.46
KIT	2.5
KIT(D816H) mutant	1.3
KIT(D816V) mutant	0.11

Q-12 exhibited obvious tendency to bind strongly in the ATP binding site of wild-type and/or mutant members of the CDK8 and KIT subfamilies, which indicated that Q-12 is an ATP competitive inhibitor of cyclin dependent kinase 8 (CDK8). Of the wild type kinases, Q-12 had the highest affinity for CDK8.

An anti-proliferative activity test was performed through screening Q-12 (Figure 1) against the NCI-60 tumor cell line panel, which revealed that Q-12 was effective at inhibiting select cell lines (Table 2). Cell lines that contained APC mutations showed apparent resistance to Q-12.

Table 2
GI 50 (μm) Values for Q-12 Against Selected Cell Line with Corresponding AKT Pathway Activation and PTEN/APC Mutation Status.

Cell Line	Q-12 GI50	APC Mutant
MDA-MB-468	0.05	No
RPMI-8226	0.31	No
NCI-H23	0.44	No
SF-295	0.31	No
HCT-116	0.27	No
BT-549	1.3	No
K-562	0.47	No
NCI/ADR-Res	0.63	No

(Table 2 Continued)

T-47D	0.71	No
KM12	12.6	Yes
SW-620	12.8	Yes
HCC-2998	15.9	Yes
HT29	15.5	Yes
COLO-205	30.4	Yes
OVCAR-3	11.2	Yes
HCT-15	19.4	Yes
SK-OV-3	25.5	Yes

Among all cell lines in NCI-60 cell panel, triple-negative breast cancer cell line, MDA-MB-468 shows the most sensitivity to Q-12 (GI50 =0.06 μM). The colon cancer cell line, HCT-116, the only colon cancer cell line without APC mutation shows high sensitivity to Q-12 (GI50 = 0.27 μM) as well.

Table 3 indicates that the activity of Q-12 is likely due to CDK8 inhibition, instead of KIT inhibition. As shown Imatinib significantly inhibits the tyrosine kinase activity of KIT, compared to Q-12. Imatinib did not have potent GI 50 value profile in HCT-116 cell line and other cell lines in NCI-60 cell panel, while Q-12 did.

Table 3

Kinase Binding Ability and GI 50 (μm) Values of C-KIT Inhibitors (Q-12 and Imatinib) Against HCT-116 Cell Line.

c-KIT inhibitor	c-KIT K_d	HCT-116 GI_{50}
Q-12	2.5	0.2
Imatinib	0.007	12.5

Breast Cancer

Breast cancer is the most common diagnosed cancer in women and also one of the leading causes of cancer death in women. Worldwide, more than 1.3 million cases of invasive breast cancer are diagnosed, and more than 450,000 women die of breast cancer each year (24). In the United States, there will be approximately 200,000 cases of invasive breast cancer and 50,000 cases of breast cancer in situ each year, and more than 40,000 women will die of breast cancer each year, the second most common cancer to lung cancer (25). Breast cancer mortality in the United States has been declining since 1990 (25). The continued decline in breast cancer mortality has been attributed to early detection of screening, improvements in adjuvant therapy, and, more recently, lower incidence rates due to the reduced use of hormone replacement therapy (26, 27). Despite the decline in incidence and mortality, breast cancer remains the leading cause of cancer death in women, accounting for 15% of all cancer deaths in women in the United States (25).

Breast cancer is highly heterogeneous and can be histologically differentiated into 21 subtypes based on cell morphology and growth. Based on distinct gene expression profiles, breast cancer can be molecularly classified into at least four subgroups with different prognosis and treatment sensitivities (28).

Peru et al. have carried out the main molecular classification of breast cancer according to the characteristics of gene mutation and protein expression by detecting the gene and protein level of breast cancer. (29). There is more than one system for molecular typing of breast cancer. The most classic one is to classify cancer cells by whether they express three proteins: ER (estrogen receptor), PR (progesterone receptor) and HER2. Depending on how positive and negative they are, different combinations are formed, which also bring about different subtypes of breast cancer. Based on receptor levels, breast cancer can be divided into luminal A, luminal B, HER2 enriched

and triple negative (30). Luminal A type of breast cancers express estrogen receptor (ER) and/or progesterone receptor (PR) but lack HER2 amplification. They are relatively less aggressive and slow growing. Compared to luminal A type, luminal B type is more aggressive (31) and also overexpress HER2. As the name indicates, HER2 enriched subtype of breast cancers have amplified HER2 expression but lack the expression of both hormonal receptors and they have poor prognosis compared to luminal subtypes (32). If a breast cancer is ER negative, PR negative, HER2 negative (ER-PR-HER2-), then we call it triple-negative breast cancer (TNBC).

TNBC accounts for approximately 10-15% of all breast cancers, and patients with TNBC have a poor prognosis compared with other breast cancer subtypes (33). Compared with hormone receptor-positive or HER2-positive disease, TNBC has a highly aggressive clinical course, earlier age of onset, greater metastatic potential, and worse clinical outcomes, manifested by higher rates of recurrence and lower survival. The molecular mechanisms driving TNBC relapse have not been fully elucidated. Thus, to date, targeted therapy has not significantly improved survival in TNBC patients, and chemotherapy remains the standard of care. Given the lack of validated molecular targets and the poor prognosis of TNBC patients, there is a clear need to deepen the understanding of TNBC at all levels and to develop better therapies.

CDK8 Substrates

Signal transducer and activator of transcription 3 (STAT3) is a member of a family of seven proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6) that relay signals from activated cytokine and growth factor receptors in the plasma membrane to the nucleus, where they regulate gene transcription (34–36). STAT3 modulates the transcription of responsive genes involved in the regulation of a variety of critical functions, including cell differentiation, proliferation, apoptosis, angiogenesis, metastasis, and immune responses (35, 37–39). Multiple lines of evidence place STAT3 at a

central node in the development, progression, and maintenance of many human tumors, validating STAT3 as an anticancer target (35, 37–40). In the cancer context, activated STAT3 is oncogenic, whereas activated STAT1 behaves as a tumor suppressor (41). Therefore, the selective inhibition of STAT3, without interference in STAT1 signaling, might be proposed as the basis for anti-cancer drug development.

In recent years, the study of nucleoprotein genes, especially the relationship between nucleoprotein genes and the occurrence and development of malignant tumors, has always been a research hotspot. The E2F1 gene belongs to the nucleoprotein gene of the transcription factor class. It is located in the 2nd band (20 q11.2) of human chromosome 20, region 11, and is about 11 kb in length. The encoded E2F1 protein is an important protein in the cell cycle. At present, 6 family members have been found, namely E2F1-E2F6, and the corresponding genes are located in different chromosomes. All E2F proteins contain a highly conserved DNA-binding region, a dimer that binds to the E2F dimer chaperone protein (DP protein) region and the activation region that binds to the RB protein family. There are two DP proteins found so far, DP21 and DP22, which combine with E2F protein to form heterodimers to enhance the transcriptional activity of E2F. E2F1, E2F2, and E2F3 proteins only bind to RB1 protein in the RB protein family, and E2F4 and E2F5 proteins only bind to RB1 protein.

Cell proliferation needs to go through four phases, of which G phase/S phase is the key point in regulating cell proliferation. E2F1 encodes a transcriptional regulator and plays an important role in the G1/S phase transition of the mammalian cell cycle. In the early stage of G1, E2F1 specifically binds to Rb to form a complex and thus its transcriptional activity is inhibited. In the middle and late stage of G1, cyclin (cyclin) binds to the corresponding cyclin-dependent kinases (CDKs), which can activate CDKs to phosphorylate Rb, and E2F1 is released and activated

from Rb protein, which promotes the cell From the G1 phase to the S phase. E2F1 is involved in a variety of physiological and pathological processes. In addition to promoting cell proliferation by transitioning cells from G1 phase to S phase, it can also induce apoptosis through p53 and p53-independent pathways. Through the p53-dependent pathway, E2F1 activates p14/p19 to inhibit the degradation of p53, and the high expression of p53 inhibits the activity of downstream CDKs, thereby interrupting the progress of the cell cycle and inducing apoptosis; through the p53-independent pathway, E2F1 regulates genes by enhancing the activities of p73, CASP-3, CASP-7, etc. induce apoptosis.

E2F1 activity has been associated with STAT3 phosphorylation. In osteosarcoma cells it has been showed that knockdown of E2F1 decreased STAT3 phosphorylation (42). There are conflicting reports in the literature on the effect of STAT3 phosphorylation on MDA-MB-468 cell viability. One report demonstrated that the nature product cosmomycin C reduced STAT3 phosphorylation and decreased cell viability in this cell line (43). The natural product reduced phosphorylation at both Ser727 and Tyr705, additionally the target of the natural product is unknown, and the authors admit that multiple mechanisms may be involved. Another report investigated the inhibition of STAT3 phosphorylation with small molecule inhibitors of the EGFR and the JAK/STAT receptor tyrosine kinase pathways to block cytokine stimulation. Decreased phosphorylation was associated with decreased cell viability (44). However, antibodies to specific phosphor-residues were not used, and inhibitors to upstream targets allow for ambiguity regarding direct cause and effect. Contrary to these reports, others have shown the opposite association. Direct treatment of MDA-MB-468 cells with relatively high concentration of EGFR (16 nM) or IL-16 family member oncostatin M (100ng/mL) cause STAT3 phosphorylation (Tyr705) reduces cell viability and induces apoptosis (PARP and caspase-3 cleavage) (45). These effects can be

reduced by STAT3 knockdown, suggesting a STAT3 dependence. It is interesting to note here that knockdown of E2F1 in in DU145 cell results in a decrease in expressions of cytokines IL-1b, TNF-a, IL-6 and IL-8 (46). E2F1 controlled cytokine expression could explain increased phosphorylation of STAT3 associated with E2F1 reactivation by CDK8 inhibition.

Aim of Study

The main aim of the study is to investigate CDK8 inhibitor Q-12 effects on CDK8 and CDK8 substrates in Triple Negative Breast Cancer cell line MDA-MB-468, in order to better understand the mechanism of anti-proliferative effect of Q-12.

Materials and Methods

Cell lines, chemicals and antibodies: MDA-MB-468 human breast cells were obtained from ATCC, USA. MDA-MB-468 were grown in Gibco® RPMI-1640 medium (Thermo Fisher Science, San Jose, CA, USA). All media were supplemented with 10% FBS (Gemini Biological Products, Calabasas, CA), 1% Antibiotic-Antimycotic mixture of Penicillin-G, Streptomycin sulfate and Amphotericin B (Thermo Fisher Science, San Jose, CA, USA). The cells were maintained in a humidified incubator at 37 °C with 5% CO2. Q-12 stock solution: 3.3 mg Q-12 into 1 mL DMSO to make 10 mM Q-12 stock solution. Antibodies to STAT1, p-STAT1 (Ser727), STAT3, p-STAT3 (Tyr705), p-STAT3 (Ser727), E2F1, CDK8and GAPDH were obtained from Cell Signaling Technology Inc (Beverly, MA, USA), anti-phospho-E2F1(Ser375) were obtained from EMD Millipore (Temecula, CA, USA), Mcl-1 and GAPDH were obtained from Santa Cruz Biotechnology Inc (Dallas, TX, USA).

Measurement of cellular viability: Cells were grown in T25 flask format at a density of 1×10 6 cells/ flask. After 24 h of culture, cells were treated with DMSO (vehicle) or 10 μM Q-12 or 10 μM OSM (Thermo Fisher Science, San Jose, CA, USA) or 10 μM Q-12 and 10 μM OSM for the indicated time period. Cell lines were subcultured by enzymatic digestion with 0.25% trypsin/EDTA solution when they reached approximately 70-80 % confluency. Floating cells and adherent cells were collected after 24 the various treatments. Cells were washed twice with 1× PBS and re-suspended in fresh media for cell viability assay. Cell viability was measured with the Muse Cell Analyzer (Millipore, Hayward, CA, USA) using the Muse Count and Viability Kit (Millipore, Hayward, CA, USA) which differentially stains viable and dead cells based on their permeability to two DNA binding dyes. Data were presented as proportional viability (%).

For viability assays, cells were plated at 2 x 10 5cells/ml in 96 well plates, in RPMI-1640 medium (Gibco) supplemented with 10% FBS and 1% Antibiotic-Antimycotic mixture of Penicillin-G, Streptomycin sulfate and Amphotericin B, and treated with increasing concentrations of S63845 (Servier), ABT-737 (Selleckchem). Cell viability was assessed using the AlamarBlue HS Cell Viability Assay (Thermo Fisher Science, San Jose, CA, USA) as per the manufacturer's instructions.

Western blotting analysis: After treatment of MDA-MB-468 with DMSO (vehicle) or 10 μM Q-12 for 24 h, cells were collected and washed twice with ice-cold 1×HBSS, then lysed in Cell Lysis Buffer (Cell Signaling Technology Inc, Beverly, MA, USA), supplement with protease inhibitor (Sigma, USA) on ice for 10 min before scrapping. Cell lysates were then centrifuged for 10 min at 13,000 rpm at 4 °C. Protein concentration of lysates was determined by BCA protein assay kit (Thermo Scientific, San Jose, CA, USA) and the lysates were adjusted with lysis buffer. Proteins were separated using SDS-PAGE and subsequently transferred to nitrocellulose membranes (Thermo Scientific, San Jose, CA, USA). The blots were blocked with 5 % BSA (20 mM Tris HCL, pH 7.5, 137 mM NaCl and 0.05 % Tween-20) at room temperature for 1 h. Incubation with specific primary antibodies was performed in blocking buffer overnight at 4°C. After washing with TBST, the blots were incubated with secondary antibody (IRDye 800 CW Donkey anti-rabbit 926-32213) for 1 h. To ensure equal protein loading, GAPDH was used as an internal control. The protein bands were detected and quantified with the Odyssey infrared imaging system (LI-COR biosciences, Lincoln, NE, USA).

siRNA mediated inhibition of gene expression: In a 12 well tissue culture plate, seed 1× 10^5 cells per well in 2 ml antibiotic-free normal growth medium supplemented with FBS. Incubate the cells at 37 °C in a CO2 incubator until the cells are 60-80% confluent. Remove media and

rinse with 1× PBS. In dark add 0.4ml transfection medium (Santa Cruz Biotechnology Inc, Dallas, TX, USA). Make a siRNA reagent by add 1.2 ml transfection media to microtube, then add 12-48 µl Control siRNA (Cell Signaling Technology Inc, Beverly, MA, USA) or STAT3 siRNA(h) (Santa Cruz Biotechnology Inc, Dallas, TX, USA), add 12-48 µl Transfection reagent (Santa Cruz Biotechnology Inc, Dallas, TX, USA), mix and incubate at room temperature for 20 min. Add 100ul siRNA reagent to each well. Incubate at incubator at 37 °C with 5% CO2 for 5 hours, directly add 0.5 µl 2 × full media to each well and incubate at incubator at 37 °C with 5% CO2 overnight. Remove media from wells and add 1 ml 1× complete media to each well. Incubate at incubator at 37 °C with 5% CO2 for 24-48 hours. Finally treated cells with DMSO (vehicle) or 10 µM Q-12.

Statistical analyses: An unpaired, student t-test was performed for the determination of significance. A p value of less than 0.05 is designated significant and is indicated by a single asterisk (*). A p value of less than 0.01 is designated significant and is indicated by two asterisks (**). A p value of less than 0.001 is designated very significant and is indicated by three asterisks (***). A p value of less than 0.0001 is designated extremely significant and is indicated by four asterisks (****).

Results

Chapter 1. CDK8 inhibitor Q-12 effects on transcription factor protein STAT3 in TNBC cell line MDA-MB-468

1.1 Q-12 effects on cancer Q-12 effects on cancer cell line viabilities and apoptosis. From GI 50 value for Q-12 against selected cancer cell lines (Table 2), Q-12 shows anti-proliferative function. To examine the anticancer activity of Q-12 on breast cancer and colorectal cancer cells in vitro, our lab's student Zhixin Lu first examined the effect of Q-12 on cell proliferation of MDA-MB-468, COLO-205 and HCT-116 cells. Next, she also investigated whether the observed growth inhibition upon Q-12 treatment was associated with induction of apoptosis. Annexin V binding was carried out using Annexin V & Dead Cell kit (Millipore). Cells were treated with DMSO or 10 μM Q-12 for 24 h. Detached and adherent cells were collected and stained with Annexin V and 7-AAD. Then the events for early and late apoptotic cells were counted with the Muse Cell Analyzer as described in Materials and Methods.

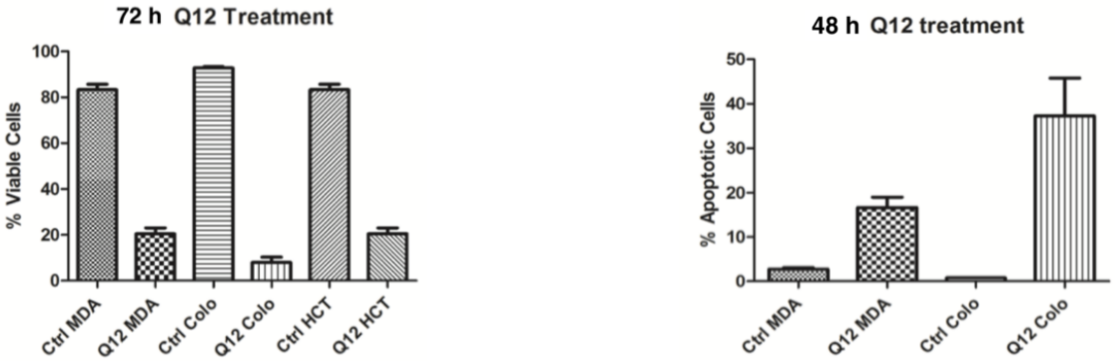

Figure 2. Cell viability and apoptosis of Q-12 treated cell lines vs control. Data represent the mean ±SD of at least three independent experiments.

Exponentially growing MDA-MB-468, COLO-205 and HCT-116 were treated with vehicle (DMSO) and 10 μM Q-12 for 72 h. Our results show that the exposure of the MDA-MB-

468, COLO-205 and HCT-116 cells to Q-12 decreased cellular viability. Cells were assayed with the Muse Cell Analyzer as described by the manufacturer. Following 24 hours of treatment, Annexin V staining was detected in cells treated with 10 μM of Q-12 (Figure 2), indicating that MDA-MB-468 and COLO-205 cells were undergoing apoptosis upon Q-12 treatment.

1.2 Q-12 effects on transcription factor proteins: β-catenin, STAT1 and STAT3. In the cell nucleus, β-catenin interacts with the TCF/LEF transcription factors to drive the transcription of WNT target genes, which is essential for β-catenin oncogenic activity (47). CDK8, as a colon cancer oncogene, has been found amplified in certain subset of colon cancers and playing an important role in the regulation of nucleus βcatenin transcription activity. STAT1, is a direct substrate of CDK8. The phosphorylation of STAT1 at Ser 727 site has been demonstrated as a pharmacodynamic biomarker of CDK8 activity (48). STAT3 plays a dual role in tumor inflammation and immunity by promoting pro-oncogenic inflammatory pathways, such as interleukin-6 (IL-6) pathway, and by involving in STAT-1 anti-tumor immune responses (49). The alternations of the balanced expression and/ or activation of STAT1 and STAT3 appears important for further developing promising compound that could regulate the STAT1:STAT3 equilibrium in the design of cancer therapeutic interventions, which lead us further studied the β-catenin, p-STAT1 (Ser 727), STAT3 expression upon CDK8 inhibitor Q-12 treatment (From our lab's previous student Zhixin Lu).

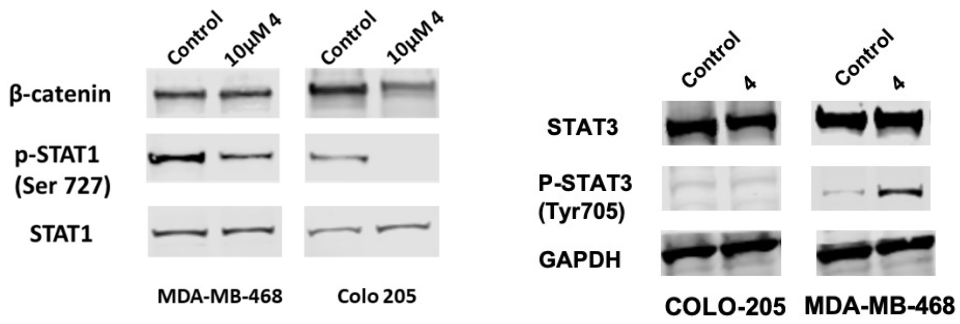

Figure 3. β-catenin, p-STAT1, STAT3 and p-STAT3 (Tyr705) expression in MDA-MB-468 and colon cancer lines: COLO-205 and HCT-116 upon 24 h10 μM Q-12 treatment. Blots in figure are represented of three independent experiments performed in triplicate.

It has been demonstrated that colon cancer cell lines treated with CDK8 RNAi result in decreased cellular levels of β –catenin (50). Likewise, treatment of colon cancer cell line Colo205 with CDK8 inhibitor Q-12 results in a dramatic depletion of β –catenin protein. The amount of β –catenin protein observed when TNBC cell line MDA-MB-468 is treated with inhibitor Q-12 did not appear to change significantly (Figure 3). The phosphorylation status of STAT1 protein is a robust pharmacodynamic marker for CDK8 inhibition (51). Treatment of the colon cancer cell line Colo 205 and the TNBC cell line with inhibitor Q-12 resulted in decreased STAT1 phosphorylation (pSTAT1), indicating inhibition of CDK8 in all these cell lines (Figure 3), as expected. In contrast, STAT3 phosphorylation (pSTAT3) status was unchanged in the Colo205 cancer cell line, while being elevated in the TNBC cell line upon treatment with CDK8 inhibitor Q-12 (Figure 3).

STAT3 is activated through tyrosine phosphorylation of Tyr705 in response to factors such as the interleukin-6 (IL-6) family cytokines, platelet-derived growth factor, and epidermal growth factor (52). STAT3 activity is primarily dependent on the level of phosphorylation at Tyr705, which is regulated by the activities of tyrosine kinases and tyrosine phosphatases specific to

STAT3 (53). Since we've observed the up-regulation of p-STAT3 (Tyr 705) expression in MDA-MB-468 upon Q-12 treatment, we want to investigate whether STAT3 activation is dependent on IL-6 family cytokines.

1.3 Q-12 and oncostatin M (OSM) effects on STAT3 and pSTAT3 (Tyr 705). It has been found that a principal mechanism of STAT3 activation in breast cancer is through the IL-6/gp130/Jak pathway (54). Direct treatment of MDA-MB-468 cells with relatively high concentration of EGF (16 nM) or IL-6 family member oncostatin M (100ng/mL) cause STAT3 phosphorylation (Tyr705) reduces cell viability and induces apoptosis. Based on that, we turned to investigate the expression of both STAT3 and pSTAT3 (Tyr 705) upon CDK8 inhibitor Q-12 treatment, OSM or co-treatment of Q-12 and OSM.

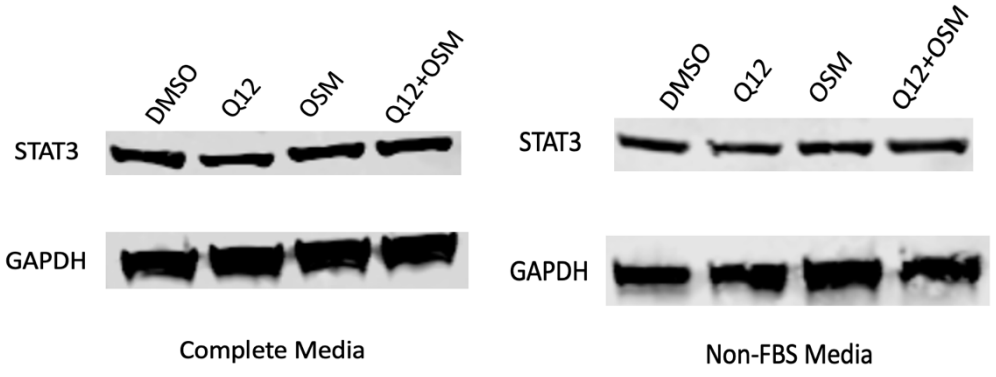

Figure 4. Effect of treatment with 10 μM OSM or10 μM Q-12 or 10 μM OSM+10 μM Q-12 (24 h) on STAT3 of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media or serum-free media. Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 1.

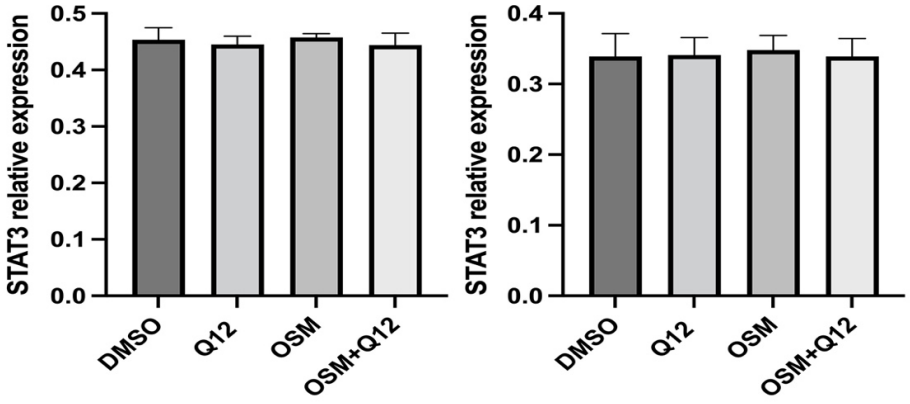

Figure 4. Effect of treatment with 10 µM OSM or10 µM Q-12 or 10 µM OSM+10 µM Q-12 (24 h) on STAT3 of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media or serum-free media. Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 1.

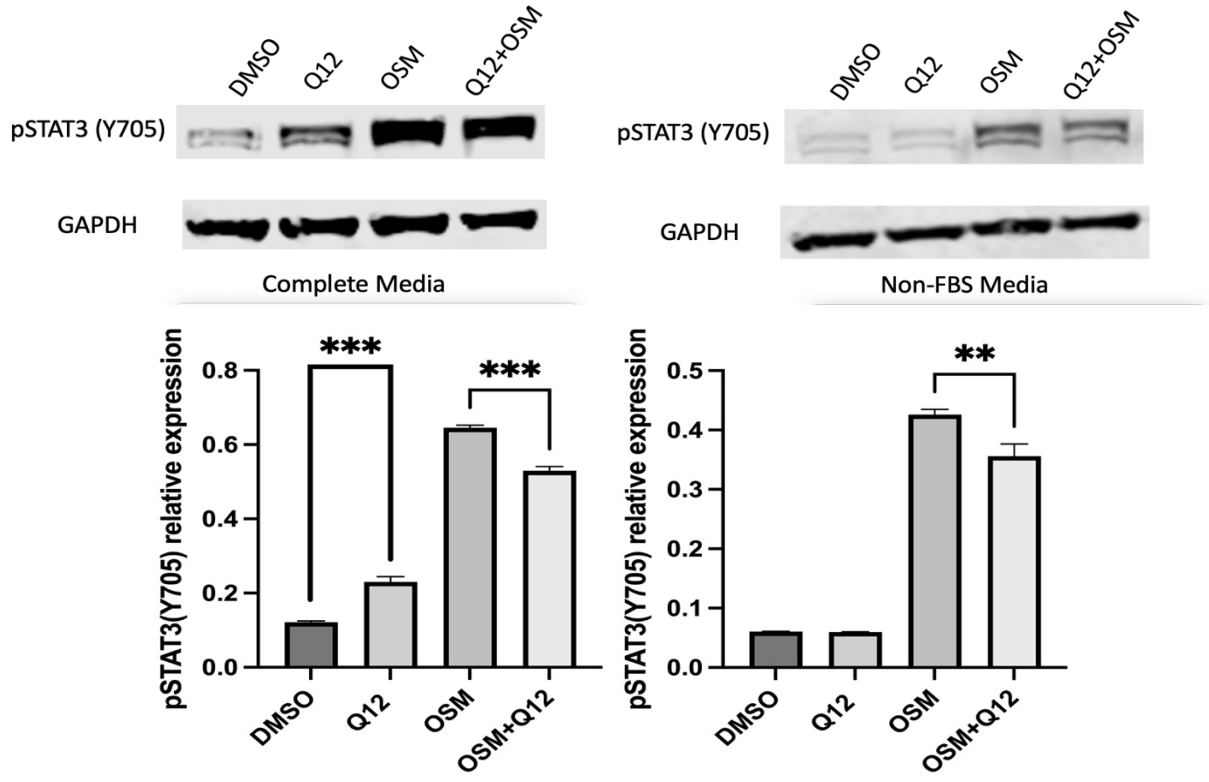

Figure 5. Effect of treatment with 10 µM OSM or10 µM Q-12 or 10 µM OSM+10 µM Q-12 (24 h) on pSTAT3 (Tyr 705) of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media or serum-free media. Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 2.

From the observation of western blotting result, there is no change as for total STAT3 expression upon Q-12 treatment, OSM treatment or co-treatment in both complete media and serum-free media in MDA-MB-468 cell lines. For p-STAT3 (Tyr 705), the expression is low and there wasn't change between the control and Q-12 treatment groups in serum-free media; but there is a definite up-regulation upon OSM treatment and co-treatment. In complete media, the expression of p-STAT3 (Tyr 705) is up-regulated upon Q-12 treatment; but there is a greater up-regulation upon OSM treatment and co-treatment.

One study (55) report that phosphorylation of Ser727 of STAT3 intrinsically, regulates the duration of STAT3 activity by promoting dephosphorylation of STAT3 pY705, which shortens the duration of transcriptional activity. They use a combination of HepG2-stat3-knockdown cells reconstituted with various STAT3 mutants and protein kinase inhibitors and showed that phospho-S727 has an intrinsic mechanism for shortening the duration of STAT3 activity, in turn shortening the duration of suppressor of cytokine signaling 3 (SOCS3) mRNA expression. They also provide evidence showing that phospho-Ser727 enhances dephosphorylation of pY705 of STAT3 largely through T-cell protein tyrosine phosphatases (TC-PTP/TC-45), and this implies the existence of an unidentified post-phospho-Ser727 process for the efficient function of TC45. This evidence leads us to further study p-STAT3 (Ser 727) expression in the MDA-MB-468 cell line upon same treatment.

1.4 Q-12 and oncostatin M (OSM) effects on pSTAT3 (Ser 727). For the experiment, we used control groups (DMSO), Q-12 treatment groups, OSM treatment groups and Q-12 and OSM co-treatment groups.

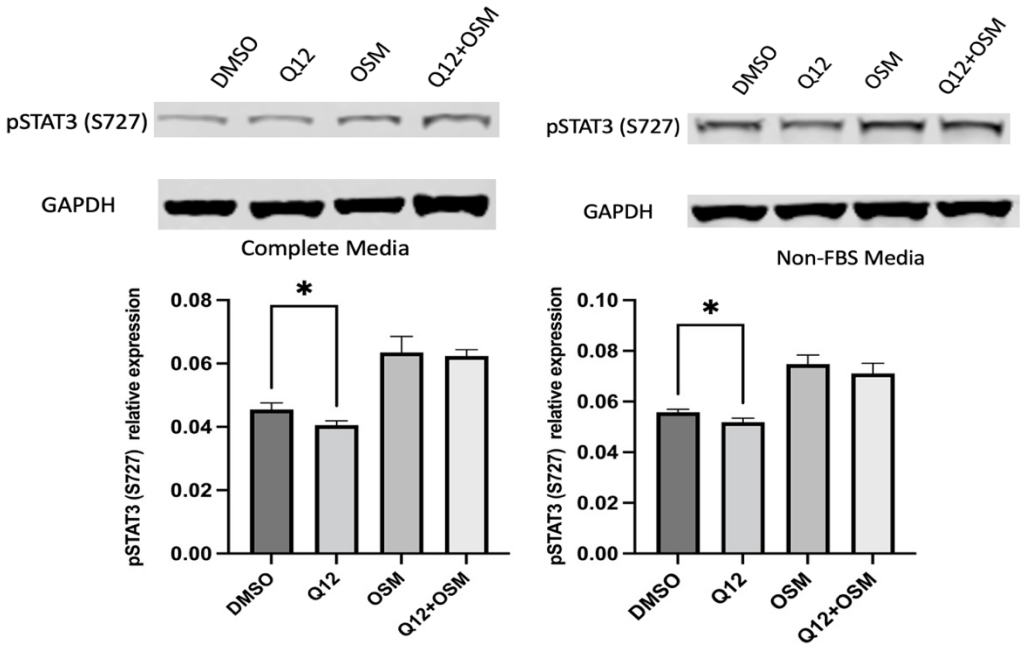

Figure 6. Effect of treatment with 10 µM OSM or10 µM Q-12 or 10 µM OSM+10 µM Q-12 (24 h) on pSTAT3 (Ser 727) of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media or serum-free media. Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 3.

From the observation of western blotting result, for p-STAT3 (Ser 727), the expression is down-regulated between the control and Q-12 treatment groups in both complete media and serum-free media; but there is a little up-regulation upon OSM treatment and co-treatment. In both complete media and serum-free media, the expression of p-STAT3 (Ser 727) is also down-regulated upon Q-12+OSM treatment groups compared to OSM treatment groups. These results suggest that Q-12 in MDA-MB468 cell line decreased p-STAT3 (Ser727) stabilized pSTAT3 (Tyr 705) cause its upregulation. Cytokines are responsible for the increased phosphorylation of STAT3 (Tyr 705). This conclusion leads us to further study and confirm p-STAT3 (Tyr 705), p-STAT3 (Ser 727) and CDK8 protein expression in both complete media and serum-free media upon Q-12 treatment.

1.5 Q-12 effects on pSTAT3 (Tyr 705), pSTAT3 (Ser 727), CDK8. For the experiment, we used control groups (DMSO), Q-12 treatment groups in both complete media and serum-free media.

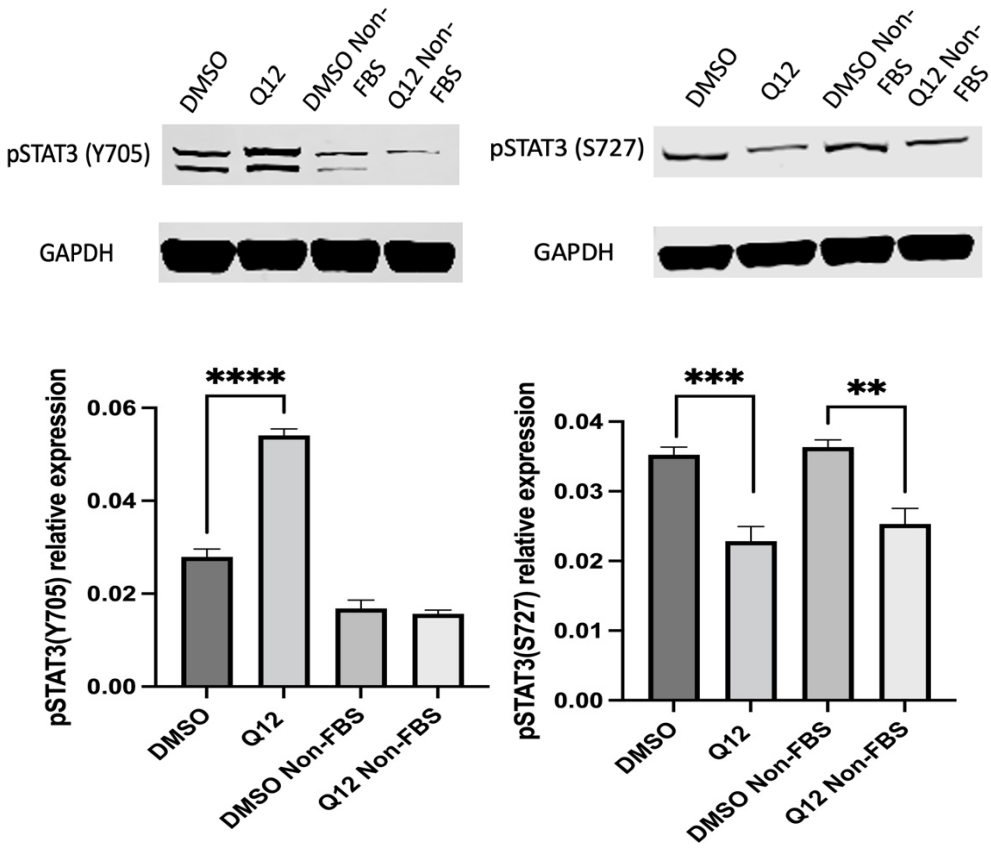

Figure 7. p-STAT3 (Tyr705) and p-STAT3 (Ser727) expression in MDA-MB-468 cell line upon 24 h 10 µM Q-12 treatment in both complete media and serum-free media. Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 4.

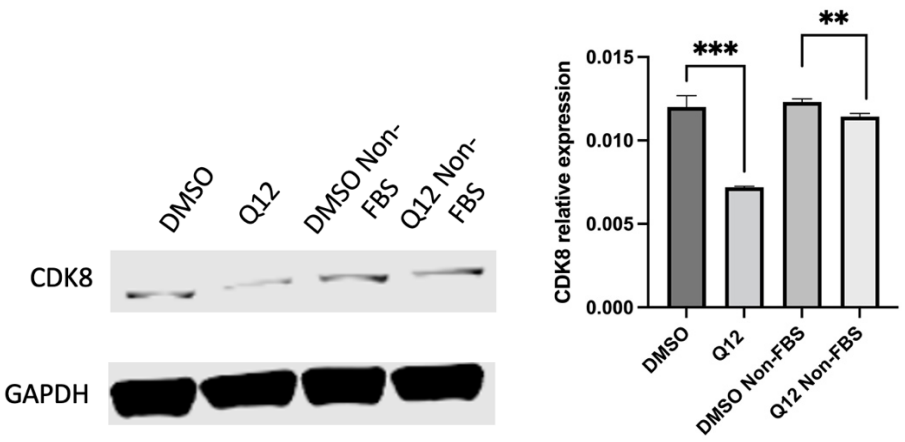

Figure 8. CDK8 protein expression in MDA-MB-468 cell line upon 24 h 10 μM Q-12 treatment in both complete media and serum-free media. Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 5.

From the observation of western blotting result, for p-STAT3 (Ser 727), the expression is down-regulated between the control and Q-12 treatment groups in both complete media and serum-free media. For p-STAT3 (Tyr 705), the expression is low and there wasn't change between the control and Q-12 treatment groups in serum-free media; In complete media, the expression of p-STAT3 (Tyr 705) is up-regulated upon Q-12 treatment. These results double confirmed that Q-12 in MDA-MB-468 cell line decreased p-STAT3 (Ser727) stabilized pSTAT3 (Tyr 705) causing its upregulation. Cytokines are responsible for the increased phosphorylation of STAT3 (Tyr 705). For the CDK8 protein, the expression is down-regulated in both complete media and serum-free media upon Q-12 treatment. This confirmed that Q-12 did reduce CDK8 protein levels no matter in complete media or serum-free media. Since we've observed the p-STAT3 (Tyr 705) and p-STAT3 (Ser 727) expression in MDA-MB-468 cells, we want to investigate whether STAT3 phosphorylation status was important for the viability effect.

1.6 Q-12 and oncostatin M (OSM) effects on MDA-MB-468 cell line viability. For the experiment, we used control groups (DMSO), Q-12 treatment groups, OSM treatment groups and Q-12 and OSM co-treatment groups in MDA-MB-468 cell line.

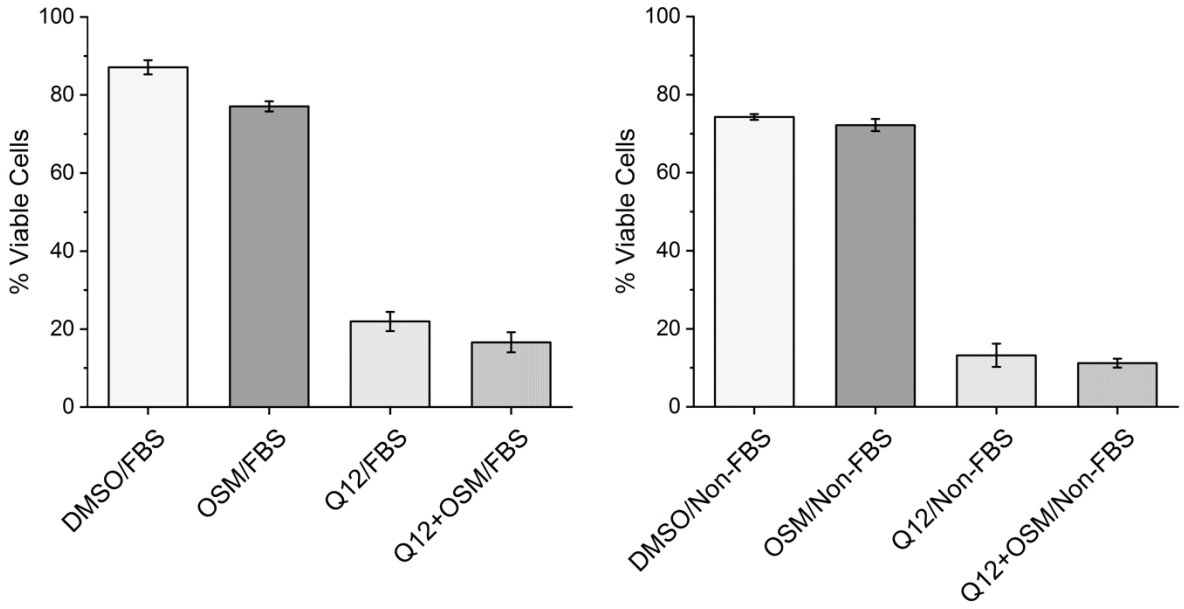

Figure 9. Effect of treatment with 10 μM OSM or10 μM Q-12 or 10 μM OSM+10 μM Q-12 (72 h) on cell viability of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media and serum-free media. Data represent the mean ±SD of at least three independent experiments.

Exponentially growing MDA-MB-468 were treated with vehicle (DMSO), 10 μM OSM, 10 μM Q-12 and 10 μM OSM+10 μM Q-12 for 72h in complete media and serum-free media. Compared to control in complete media, MDA-MB-468 cell viability with 10 μM OSM alone had a significant decrease in complete media while in serum-free media had an nonsignificant decrease much, which indicating that STAT3 activation did decrease cell viability in MDA-MB-468. The 10 μM Q-12 treatment group, co-treatment of 10 μM OSM and 10 μM Q-12 had more cell death than OSM alone group in both complete media and serum-free media, which indicates that Q-12 induce cell death was more pronounced than treated with OSM alone. These results show that Q-

12 can decrease cell viability more significantly than the activation of STAT3 alone, suggesting that STAT3 activation may not play a major part in the Q-12-mediated killing of MDA-MB-468 cells. In all, Q-12 effects on other CDK8 substrates may play an important role to decreasing cell viability in MDA-MB-468 cell line.

1.7 Discussion. From the above results of the expression of STAT3, p-STAT3 (Tyr 705) and p-STAT3 (Ser 727) in MDA-MB-468 cells upon treatment with Q-12, OSM and co-treatment of Q-12+OSM, we concluded that Q-12 decrease p-STAT3 (Ser727) stabilized p-STAT3 (Tyr 705) cause its upregulation. Cytokines is responsible for the increased phosphorylation of STAT3 (Tyr 705). STAT3 activation can also occur through interleuin-6 (IL-6), epidermal growth factor, vascular endothelial growth factor, etc.(56) IL-6 was shown to serve as an activator of STAT3 in breast cancer. A positive correlation between IL-6 and p-STAT3 expression levels was found in primary breast cancers and IL-6 family of cytokines (54).

The limitation of this part of the experiment is that we also need to verify that the activation site of OSM is pSTAT3 (Y705). We also need to determine the relationship between STAT3 phosphorylation and MDA cell activity and apoptosis. We could only phosphorylate STAT3 at site Y705 and only phosphorylate STAT3 at site S727 to measure cell activity and apoptosis. We can also determine the effect on cell viability and apoptosis of STAT3 knockdown of CDKB inhibitor treated cells and effect on cell viability and apoptosis of STAT3 knockdown on IL-6 treated cells. We can evaluate at least 2 siRNA targeting the open reading frame of STAT3 mRNA and a negative control siRNA for ability to knockdown expression of STAT3 protein (detected by western blot). We can also perform the OSM stimulation to STAT3 knockdown cells in serum free media, to ensure stimulation is due solely to IL-6.

In 2016, a study (57) confirmed the existence of a feedback loop: ILK-Akt-NF-kB-IL-6. This signal loop was shown to activate E2F1 expression through STAT3/cyclin D1/CDK2 signaling. Additionally, Q-12 can decrease cell viability to a greater extent than the activation of STAT3 alone, suggesting that STAT3 activation may not plays a major part in the Q-12-mediated killing the MDA-MB-468 cells. E2F1 is a well-known cell cycle regulator and CDK8 is one of the important kinases which negatively regulates E2F1. It also has been shown (58) that there is a direct link between E2F1 and apoptotic machinery, especially the expression level of the pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins.

Since Q-12 is affecting gene transcription of transcription factor STAT3, we further investigated the other transcription factors that may involve in Q-12 modulation.

Chapter 2. CDK8 inhibitor Q-12 effects on transcription factor protein E2F1 and apoptosis protein Mcl-1 in TNBC cell line MDA-MB-468

2.1 Q-12 effects on MDA-MB-468 cell line inhibitor of apoptosis and pro-survival Bcl-2 protein expression. From previous study in our lab, to further understand the role of Q-12 in apoptosis in MDA-MB-468 and COLO-205 cell lines, Western blotting was used to detect inhibitors of apoptosis proteins (IAP) family and pro-survival Bcl-2 family in non-treated cancer cells. We found out that the expression of Survivin, XIAP, Bcl-2 and Mcl-1 are relatively high in MDAMB-468 cell line, so we chose these proteins to investigate further with Q-12 treatment. We focused on Survivin, XIAP, Bcl-2 and Mcl-1 in MDA-MB-468 cell line upon 10 μM Q-12 treatment to see how Q-12 affected those protein expressions.

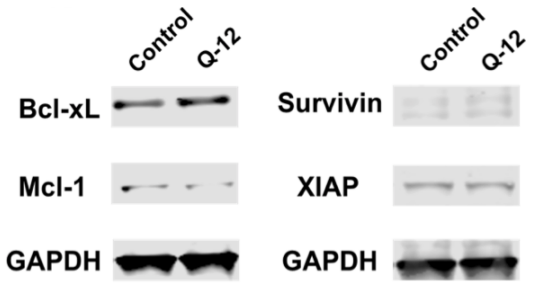

Figure 10. Bcl-xL, Mcl-1, Survivin and XIAP expression in MDA-MB-468 cell line upon 24 h 10 μM Q-12 treatment. Blots in figure are represented of three independent experiments performed in triplicate.

Among all those high expressed IAP and pro-survival Bcl-2 family members, we found out that the expression of Mcl-1 was downregulated upon Q-12 treatment in MDA-MB-468 (30% decrease) cell lines, which indicates Q-12 can modulate the expression Mcl-1 in those cell lines. This evidence leads us to investigate whether CDK8 substrates can modulate Mcl-1 expression.

2.2 Mcl-1 expression upon STAT3 knockdown with Q-12. For this experiment, we want to investigate whether STAT3 can modulate Mcl-1 expression. We use STAT3 siRNA(h) (Santa Cruz Biotechnology Inc, Dallas, TX, USA) to knockdown STAT3 upon DMSO treatment in MDA-MB-468 cell line. We also used wild type MDA-MB-468 cells treated with DMSO and Q-12.

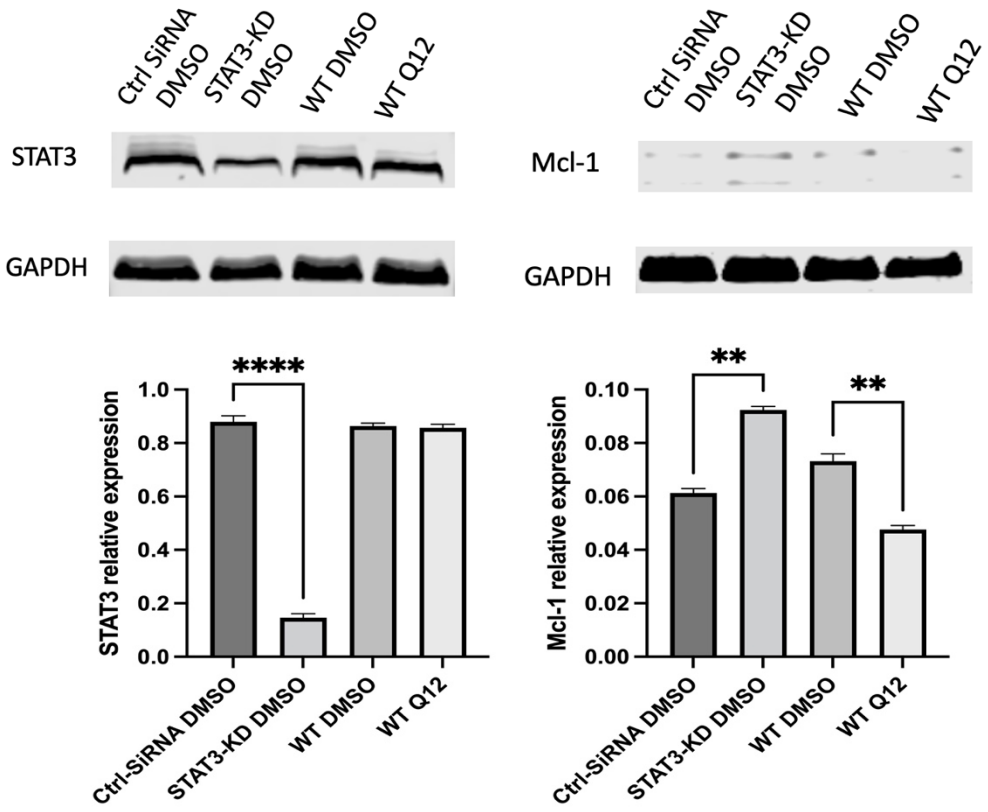

Figure 11. Effect of STAT3 knockdown (24 h) on STAT3 and Mcl-1 of MDA-MB-468 cells compared to wild type cells treated with DMSO (10 μM). Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 6.

From the observation of western blotting result, STAT3 expression is downregulated upon STAT3 siRNA inhibition of gene expression compared to control siRNA group or wild type MDA-MB-468 cells group with the treatment of DMSO or Q-12. For Mcl-1, the knockdown of STAT3

increase anti-apoptotic protein Mcl-1 expression. And Q-12 did downregulate the Mcl-1 expression in wild type MDA-MB-468 cells compared to wild type control (DMSO) group, which double confirmed Q-12 can modulate the expression Mcl-1 in MDA-MB-468 cell line. This evidence leads us to further investigate whether knockdown of STAT3 will affect the cell viability in MDA-MB-468 cell line.

2.3 STAT3 knockdown experiment – Cell Viability. For the experiment, we used control siRNA groups (DMSO) and STAT3 knockdown groups (DMSO) in MDA-MB-468 cell line upon 72h treatment.

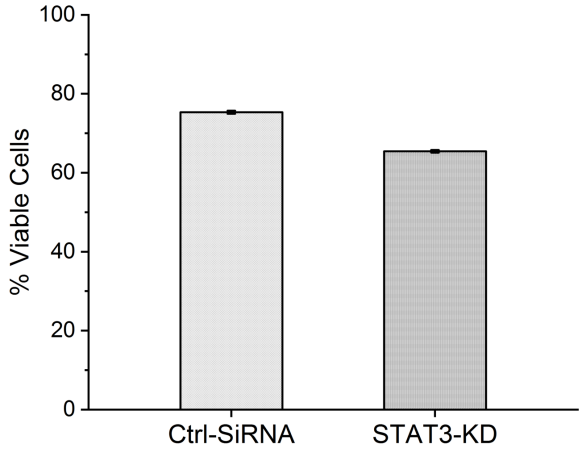

Figure 12. Effect of STAT3 knockdown (72 h) on cell viability of MDA-MB-468 cells compared to control siRNA treated with DMSO (10 μM). Blots in figure are represented of three independent experiments performed in triplicate.

Compared to control siRNA group, the MDA-MB-468 cell viability with STAT3 knockdown had a significant decrease, but not as large a decrease as Q-12 treatment. These results suggest that STAT3 may not play a major role in the Q-12-mediated killing the MDA-MB-468 cells. There must be some other CDK8 substate regulate the Mcl-1 expression to affect cell viability. Since E2F1 is a well-known cell cycle regulator and CDK8 is one of the important

kinases which negatively regulates E2F1, we further studied the expression of E2F1 upon Q-12 treatment.

2.4 Q-12 effects on transcription factor E2F1 and pE2F1 (Ser 375). One study (59) reported that CDK8 phosphorylates serine 375 in E2F1 both in vitro and in cells, and that the phosphorylation is dependent on CDK8 kinase activity. The phosphorylation of S375 by CDK8 regulates E2F1 ability to repress transcription of β-catenin/TCF-dependent genes, as well as activation of E2F1-dependent genes. This evidence led us to further study the E2F1 and p-E2F1 (Ser 375) expression upon Q-12 treatment over time in MDA-MB-468 cell line.

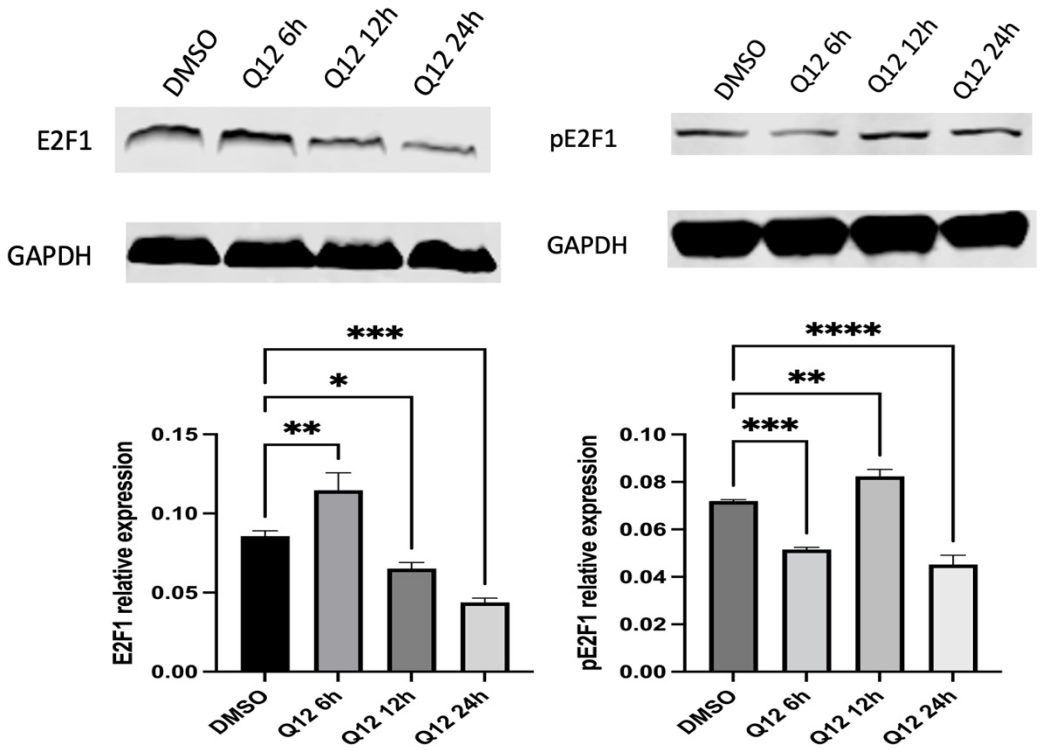

Figure 13. Effect of treatment with 10 µM Q-12 (6,12,24 h) on E2F1 and p-E2F1 (Ser 375) of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells. Blots in figure are represented of three independent experiments performed in triplicate. Full gel is at Appendix Figure 7.

From the observation of western blotting result, the p-E2F1 (S375) expression is downregulated upon Q-12 treatment at 6h compared to control group (DMSO). For E2F1, the expression is up regulated upon Q-12 treatment at 6h. One study (60) reported that the effect of E2F1 overexpression in two breast cancer cell lines, MDA-MB-436 and MDA-MB-468, which lack pRb and functional p53, was accumulation of cells in G2/S phase and apoptosis.

But with the time increase, the E2F1 expression is downregulated upon Q-12 treatment at 12h and 24h compared to 6h. E2F1 expression is cell-cycle regulated, one study (61) showed that processing of E2F1 through a ubiquitin-dependent proteolytic pathway. They also demonstrated that complex formation with pRB blocks E2F1 degradation suggesting at least one means through which E2F1 turnover is modulated. This evidence explains the observed activation of E2F1 followed by a gradual decrease in degradation over time. Direct repression of the Mcl-1 promoter by E2F1 (62), this evidence leads us to further study Mcl-1 inhibitor affect cell viability in MDA-MB-468 cell line.

2.5 Mcl-1inhibitor experiment – Cell Viability. ABT-737 is a BH3 mimetic inhibitor of Bcl-xL, Bcl-2 and Bcl-w with EC_{50} of 78.7 nM, 30.3 nM and 197.8 nM in cell-free assays, respectively; no inhibition observed against Mcl-1, Bcl-B or Bfl-1. ABT-737 induces mitochondrial pathway apoptosis and mitophagy. S63845 is a new, selective MCL-1 inhibitor with the Kd value of 0.19 nM and has no discernible binding to the other BCL-2 members, BCL-2 or BCL-XL. We examined the cell viability of MDA-MB-468 cells upon 72 h treated with increasing concentrations of S63845, ABT-737.

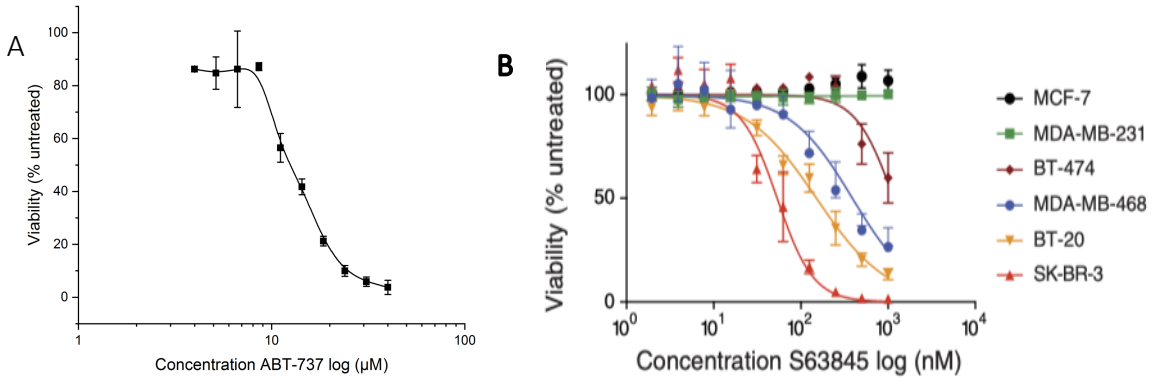

Figure 14. Cell viability analysis after increasing concentrations of S63845 or ABT-737 monotherapy. (A) MDA-MB-468 cell lines were treated at increasing concentrations of ABT-737 for 72 hours before assessment of viability using alamarBlue HS. Means ± SEM for n ≥ 3 independent experiments are shown. (B) Cell lines were treated at increasing concentrations of S63845 for 24 hours before assessment of viability using CellTiter-Glo. Means ± SEM for n ≥ 3 independent experiments are shown. (63)

From the observation of the figure, the IC_{50} of ABT-737 is around 10 μM while the IC_{50} of S63845 is 500 nM in MDA-MB-468 cell lines. These results suggest that MCL-1 is an important survival factor in MDA-MB-468 cell lines. And suggest that MCL-1 could be an important survival factor and therapeutic target in TNBC tumors.

2.6 Discussion. From the above results of the expression of Mcl-1, E2F1 and p-E2F1 (S375) in MDA-MB-468 cells upon Q-12 treatment, we concluded that Mcl-1 and E2F1 were modulated by Q-12 and the decrease of p-E2F1 (S375) induce the E2F1 activation in MDA-MB-468 cells. STAT3 was not a major factor to decrease the cell viability and induce of apoptosis, which means E2F1 may play an important role in MDA-MB-468 cell line. The decrease of Mcl-1 expression may be due to the E2F1 activation upon Q-12 treatment in MDA-MB-468.

The limitation of this chapter is we need to link CDK8 inhibition to E2F1 reactivation. We can see both effect of CDK8 knockdown on E2F1 protein and effect of CDK8 knockdown on E2F1 transcribed gene products to confirm this hypothesis. We can investigate the effect of CDK8

knockdown on the status of both total and phosphorylated E2F1 protein, we can also knockdown CDK8 and look for an effect on E2F1 transcribed genes. We can use RNA interference to block the E2F1 message in the presence of CDK8 inhibition and use western blot technique to observe changes in STAT3 phosphorylation (Tyr705) and STAT3 total protein and E2F1 to confirm our hypothesis.

Programmed cell death (PCD) plays an important role in determining cancer cell fate. Apoptosis, autophagy, and programmed necrosis are the three main forms of PCD, which could be distinguished by their morphological differences (64). When BH3-only proteins are induced or activated, they interact and pair with core Bcl-2 family proteins, such as Mcl1, BAX, BAK, to promote apoptosis. BH3-only proteins can be activated posttranslationally and their expression can also be induced by transcription factors (65).

One study (60) determined that the effect of E2F1 overexpression in two breast cancer cell lines, MDA-MB-436 and MDA-MB-468, which lack pRb and functional p53, was accumulation of cells in G2/S phase and apoptosis. pRb plays an important role in regulating the proliferative and apoptotic effects of E2F1; however, the role of pRb in E2F1 mediated apoptosis is cell context dependent. While another report (61) found that pRB blocks ubiquitination and stabilizes E2F1. Therefore, modulation of E2F1 could selectively induce apoptosis in breast cancer cells that lack pRb, while sparing normal cells.

The above experimental evidence and literature presented suggest that Q-12 inhibits E2F1 phosphorylation and promotes E2F1 activation, which results in modulating the expression of key anti- and pro-apoptotic proteins Mcl-1. E2F1 may play an important role in apoptosis process happened in MDA-MB-468 cell line upon Q-12 treatment.

Conclusions

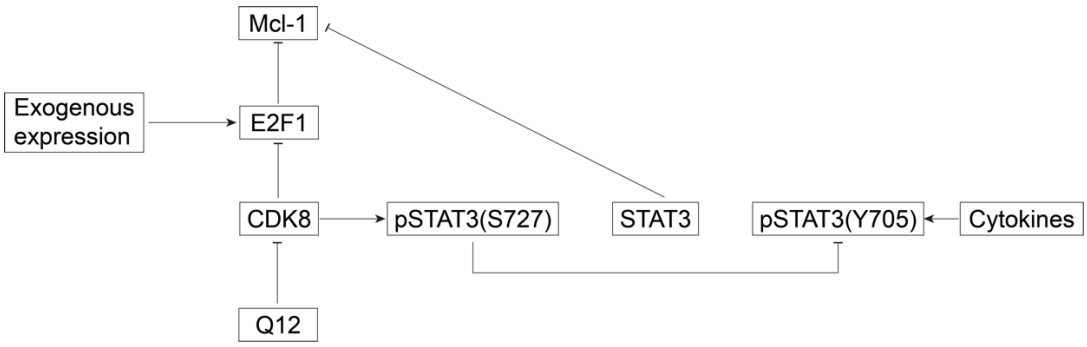

Figure 15. Schematic illustration of potential role of CDK8 inhibitor Q12 effects on CDK8 and CDK8 substrates in triple negative breast cancer cell line MDA-MB-468.

In MDA-MB-468 and COLO-205 cell lines, Q-12 modulated transcriptional factor p-STAT1 reduction. In colorectal cancer cell line, COLO-205, Q-12 induced β-catenin reduction, but not in triple negative breast cancer cell line, the MDA-MB-468. The expression of p-STAT3 (Ser727) was modulated by Q-12 in MDA-MB-468 cell line only. Q-12 decrease p-STAT3 (Ser 727) stabilized p-STAT3 (Tyr 705) cause its upregulation. Cytokines is responsible for the increased phosphorylation of STAT3 (Tyr 705). Q-12 inhibit phosphorylation of CDK8 substrates STAT3 (Ser 727), STAT1 (Ser 727), E2F1 (Ser 375) and reduce CDK8 protein levels. Q-12 initially increase E2F1 protein levels and Q-12 also decrease Mcl-1protein levels.

CDK8 regulates gene transcription as a part of the mediator complex. CDK8 can also directly phosphorylate transcription factors E2F1 and STAT1. Study (66) have shown that CDK8 as a negative regulator of STAT3 transcriptional activities, which interacts with STAT3 upon IL-6 stimulation. Inhibition of CDK8 activity, using specific small molecule inhibitors, reduces the IL-6-induced phosphoproteome by 23% in human CD4 +T helper 1 (Th-1) cells, including STAT3 S727 phosphorylation. Our results indicated that STAT3 activation may not play an important role in Q-12 regulation in MDA-MB-468 cell line.

One study (60) reported that CDK8 phosphorylates serine 375 in E2F1 both in vitro and in cells, and that phosphorylation of this residue is required for E2F1 interaction with CDK8, and that the phosphorylation is dependent on CDK8 kinase activity. The phosphorylation of S375 by CDK8 regulates E2F1 ability to repress transcription of b-catenin/TCF-dependent genes, as well as activation of E2F1-dependent genes. Whether E2F1 is the key regulator in the apoptosis process induced by Q-12 and how Q-12 modulates E2F1 expression still needs to be further investigated. One way to probe E2F1 42 engagement upon Q-12 treatment in MDA-MB-468 cell line is to use small interfering RNA to prevent the re-activation of E2F1. SiRNA of E2F1 will interfere with the expression of E2F1 genes with complementary nucleotide sequences by degrading its mRNA after transcription. If knocking down E2F1 protects the cell from Q-12, which will suggest that E2F1 activation would be critical for the apoptosis process in the MDAMB-468 cells upon Q-12 treatment. Another study (62) also show that E2F1 potently represses the expression of Mcl-1 - an anti-apoptotic Bcl-2 family member whose depletion results in apoptosis. These evidence confirmed our results Q-12 initially increase E2F1 protein levels and Q-12 also decrease Mcl-1protein levels.

To study the mechanisms of anti-proliferative effect of Q-12 is an important and necessary step to further identify the relationship among CDK8, E2F1 and Mcl-1 in MDA-MB-468 cell line, these studies will identify a lot of new potential targets for cancers.To better understand the effects of E2F1 and Mcl-1 on CDK8 substrates will link E2F1 to decrease viability and induction of apoptosis in MDA-MB-468 cells, and it can help us better understand the mechanism of Q-12.

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APPENDIX A: FULL GEL WESTERN BLOTTING IMAGES

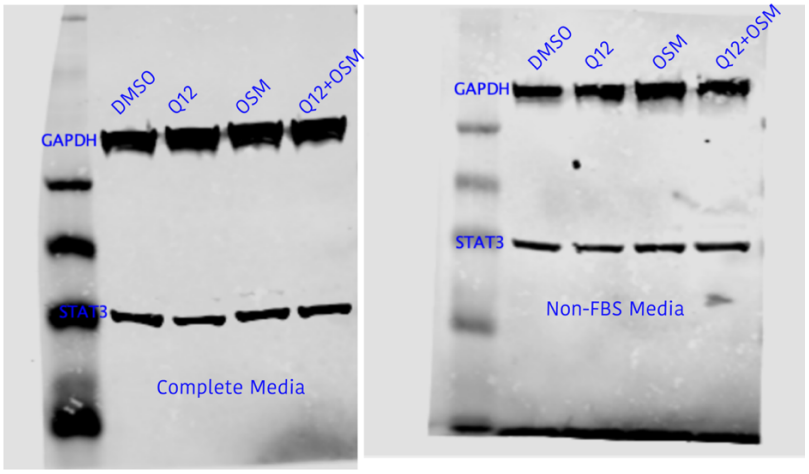

Figure 1. Effect of treatment with 10 µM OSM or10 µM Q-12 or 10 µM OSM+10 µM Q-12 (24 h) on STAT3 of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media or serum-free media. Blots in figure are represented of three independent experiments performed in triplicate.

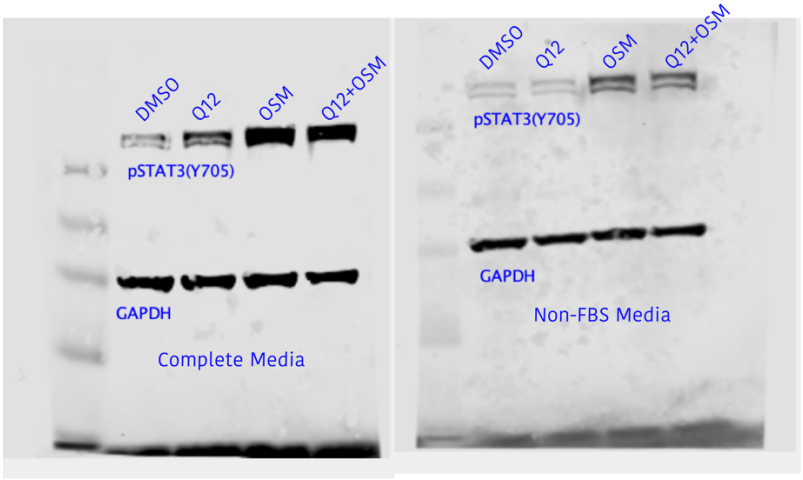

Figure 2. Effect of treatment with 10 µM OSM or10 µM Q-12 or 10 µM OSM+10 µM Q-12 (24 h) on pSTAT3 (Tyr 705) of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media or serum-free media. Blots in figure are represented of three independent experiments performed in triplicate.

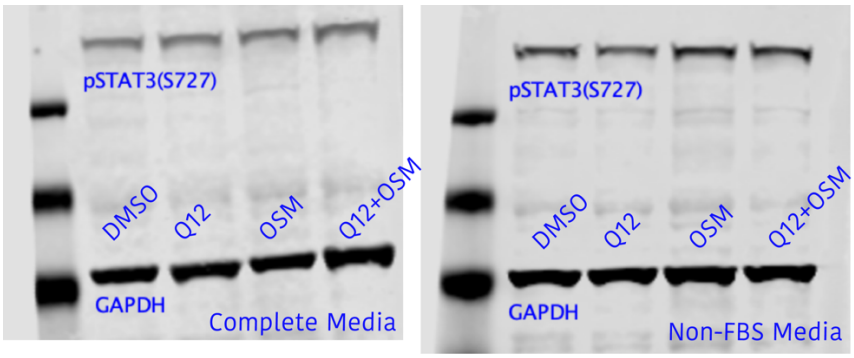

Figure 3. Effect of treatment with 10 µM OSM or10 µM Q-12 or 10 µM OSM+10 µM Q-12 (24 h) on pSTAT3 (Ser 727) of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells in complete media or serum-free media. Blots in figure are represented of three independent experiments performed in triplicate.

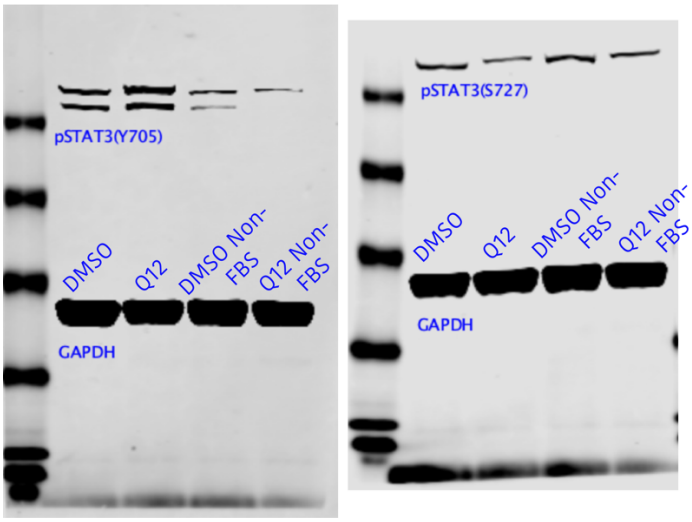

Figure 4. p-STAT3 (Tyr705) and p-STAT3 (Ser727) expression in MDA-MB-468 cell line upon 24 h 10 µM Q-12 treatment in both complete media and serum-free media. Blots in figure are represented of three independent experiments performed in triplicate.

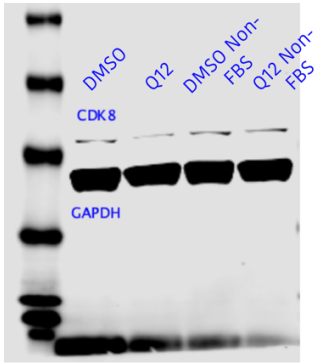

Figure 5. CDK8 protein expression in MDA-MB-468 cell line upon 24 h 10 µM Q-12 treatment in both complete media and serum-free media. Blots in figure are represented of three independent experiments performed in triplicate.

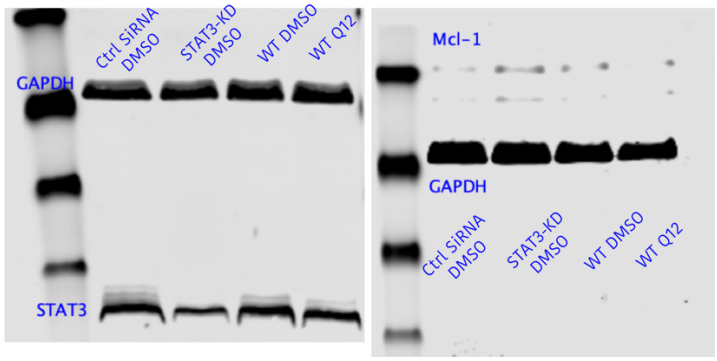

Figure 6. Effect of STAT3 knockdown (24 h) on STAT3 and Mcl-1 of MDA-MB-468 cells compared to wild type cells treated with DMSO (10 µM). Blots in figure are represented of three independent experiments performed in triplicate.

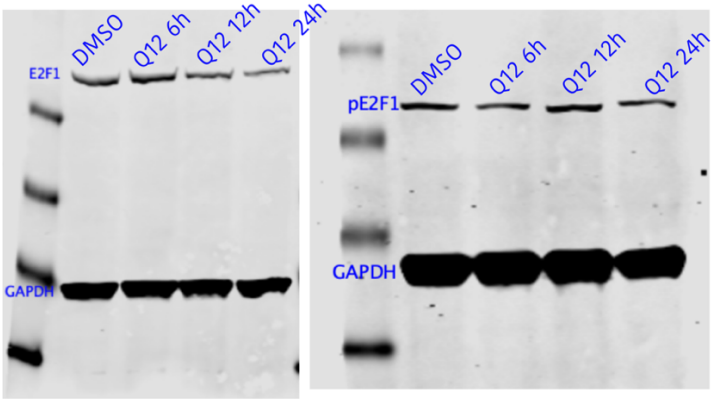

Figure 7. Effect of treatment with 10 µM Q-12 (6,12,24 h) on E2F1 and p-E2F1 (Ser 375) of MDA-MB-468 (MDA) cells compared to vehicle-treated control (DMSO) cells. Blots in figure are represented of three independent experiments performed in triplicate.