ผลของกรดไขมัน 10-hydroxy-2decenoic acid (10-HDA) ต่อการเพิ่มจำนวนและการแพร่กระจายของเซลล์มะเร็งเต้านม

THE INHIBITORY EFFECT OF 10-HYDROXY-2DECENOIC ACID (10-HDA) ON BREAST CANCER CELL PROLIFERATION AND METASTASIS

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สุกัญญา เพ็งพานิช

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SUKANYA PENGPANICH

A Dissertation Submitted in partial Fulfillment of Requirements

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THE DISSERTATION TITLED

THE INHIBITORY EFFECT OF 10-HYDROXY-2DECENOIC ACID (10-HDA) ON BREAST CANCER CELL PROLIFERATION AND METASTASIS

BY

SUKANYA PENGPANICH

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES AT SRINAKHARINWIROT UNIVERSITY

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Author	SUKANYA PENGPANICH
Degree	DOCTOR OF PHILOSOPHY
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Thesis Advisor	Assistant Professor Wanlaya Tanechpongtamb , Ph.D.

Due to the fact that breast cancer is still a major problem for women all around the world, so this work would like to discover of a new type of breast cancer treatment, especially new anticancer agent. Since natural compounds have received attention worldwide as valuable sources for anticancer drug development and for this reason, to this aim 10-hydroxy-2-decenoic acid (10-HDA), which was extracted from royal jelly (RJ) was selected to investigate the effects of breast cancer proliferation and metastasis in this study. Several types of aggressive breast cancer with a high rate of metastasis were used as models to compare to non-cancerous models. The cytotoxic effect of 10-HDA on four types of breast cancer cells; MDA-MB231, MDA-MB436, HCC1937 and MCF-7 were shown the IC_{50} at 3.5, 5.1, 5.26, 5.22 mM respectively, while in the normal human epithelial breast cell (MCF-10a), African green monkey cell (Vero) and fibroblast NIH3T3 (wt) and mutant forms of $BRAF^{V600E}$ were IC_{50} at 5, 18.5, 10 and 7 mM respectively. Additionally, 10-HDA significantly inhibited cell migration, adhesion and invasion and VEGFA-induced angiogenesis. Interestingly, 10-HDA could inhibit the phosphorylation of FAK, which leads to restraining the activation of PI3-K, RAC1 and ERK1/2, resulting in the blockage of cell migration and invasion. However, we found that AKT activation was not dependent on PI3-K signaling, which may imply the possibility of apoptosis induction. The inhibiting of FAK/PI3-K/RAC1 signaling was shown to relate to the inhibition of co-localization of vinculin and actin cytoskeleton on focal adhesion to supporting the inhibition effect of 10-HDA on cancer cell migration. Therefore, it can conclude that 10-HDA has the ability to inhibit breast cancer cells proliferation and metastasis. Importantly, this work firstly demonstrated the effect of 10-HDA on several types of aggressive breast cancer cells that are normally resistant to treatment. The development of 10-HDA as an anti-metastatic agent would be useful for more successful breast cancer treatment.

Keyword : 10-HDA, Breast cancer, Cell proliferation, Metastasis, Angiogenesis, Focal adhesion

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CHAPTER I

INTRODUCTION

Breast cancer is the most frequent cancer in women worldwide. The data from GLOBOCAN 2012 (International Agency for Research on Cancer, IARC) reported that the new breast cancer cases are estimated at 1.7 million or 25.2% and mortality rate of 522,000 people as percentage at 14.7% [1]. In Thailand, the incidence of breast cancer in the year 2012 was at 13,653 per 100,000 women as percentage of 22.4 % and mortality rate at 5,092 people or 13.8% [2]. Breast cancer cells can be divided as 2 main types which are 1) positive receptors (estrogen, progesterone and HER/neu receptor) this type can be treated by hormone therapies such as tamoxifen, aromatase inhibitor and Herceptin for interfering with the HER2/neu receptor, and 2) negative receptors which do not have estrogen, progesterone and HER/neu receptor, so this type can be called as triple negative receptors (TNBC). TNBCs tend to aggressive and rapidly growing, so they are hardly treated by traditional methods, hormone therapies or target therapy. The current treatments of TNBCs use the combination of surgery, radiotherapy and chemotherapy; however, all these treatments are not so effective. Therefore, TNBCs' patient died from the progressive of diseases into the late metastatic stage [3, 4].

At present, natural compounds are received much attention from all nations as resources for anticancer drugs development. Many of anticancer drugs available nowadays are produced from natural agents. Therefore, this research aimed to produce the anticancer drug from natural resources against breast cancer and focused specifically to the inhibition of the metastasis of aggressive breast cancer types that have difficulty in treatment. Ten-hydroxy-2-decenoic acid (10-HDA), a major fatty acid compound extracted from royal jelly was selected for studying as this compound has

various of biological activites such as anti-oxidant, anti-bacterial, anti-inflammation etc. Importantly, Izuta et.al, 2009 reported that 10-HDA could inhibit cell proliferation, cell migration and angiogenesis in HUVEC cells [5]. Then, it is possible that 10-HDA may inhibit the processes related to breast cancer cell proliferation and metastasis either migration, invasion or adhesion. In addition, this work also interested to examine the effect of 10-HDA on the activity of focal adhesion kinase (FAK) or protein tyrosine kinase 2 (PTK2). FAK is normally a prominent molecule localized at focal adhesion (FA), or the adhesive contact where the cell uses integrin receptors to link with extracellularmatrix (ECM) [6]. Phosphorylation of FAK regulates actin cytoskeleton remodeling, cell growth and survival through downstream signaling of PI3K, AKT, RAC1, JNK, and p38 [7]. Hence, FAK is associated with cellular adhesion and spreading processes. In breast cancer cells, it has been shown that when FAK was blocked, cancer cells became less metastatic and decreasing in mobility [8]. Thus, FAK is considered as one promising therapeutic target for cancer treatment. Apart from FAK, this work would like to determine the effect of 10-HDA on the releasing of vascular endothelial growth factor (VEGF). As it is known that, VEGF is an important factor to stimulate angiogenesis. VEGF is required for cancer survival in the distance site [9]. The VEGF family comprises of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) [10]. VEGF-A is mostly studied and defined as a component of the pre-metastatic niche in the target organs, while VEGF-C and VEGF-D are involved in lymphangiogenesis [11]. VEGF-A was then selected as one marker for monitoring the effect of 10-HDA on angiogenesis.

In summary, this work to demonstrates whether 10-HDA has the ability to inhibit aggressive breast cancer cell proliferation and metastatic processes. In addition, the function of FAK at FA, the formation of FA, and the releasing of VEGF-A were investigated. As shown in the results, this work for the first time demonstrated that 10-HDA has the effect to inhibit proliferation, metastasis processes, focal adhesion

formation, the phosphorylation of FAK and subsequent of its downstream targets (PI3K, AKT, RAC1, JNK, p38 and ERK) and the expression of VEGF-A. These data indicated that 10-HDA has the ability to control breast cancer progression to the metastatic stage and might be possible for apoptosis induction through the FAK and MAPK pathway. Therefore, 10-HDA is an interested compound to treat on TNBCs and may be an effective substance to develop as anticancer agent which may increase the possibility of patient survival. Although this work is the first examined of 10-HDA on breast cancer cells, other experiments need to be further investigated.

Objectives

To investigate the inhibitory effects of 10-HDA on breast cancer cells by determination on

1. Cytotoxic effect
2. Metastatic processes
3. VEGF secretion
4. Focal adhesion formation
5. Phosphorylation of FAK and its downstream targets (PI3K, AKT, RAC1, JNK, p38 and ERK)

Hypothesis

10-HDA, a purified compound of royal jelly, may act as anti-metastasis properties on a highly metastatic breast cancer cells by inhibiting cell migration, invasion, adhesion, VEGF-induced angiogenesis, focal adhesion formation and the signaling pathway involved.

CHAPTER II

LITERATURE REVIEW

Breast cancer

Breast cancer statistics

In the year 2012, The International Agency for Research on Cancer (IARC) which is the specialized cancer agency of the World Health Organization has been reported the data on cancer incidence, mortality, and prevalence worldwide on the online database, GLOBOCAN 2012. The latest world cancer statistics show that the global cancer burden rises to 14.1 million new cases and 8.2 million cancer deaths in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. Breast cancer is the world's second most common cancer diagnosed subordinate to lung cancer. In female worldwide, breast cancer is the most frequent cancer occurred with 1.7 million women and also the most common cause of cancer death among women, which were 522,000 deaths in the year 2012 (Fig 1-2) [(12)]. In Thailand, breast cancer is the most common cancer in women with the incidence of 13,653 cases per 100,000 women and mortality rate was 5,092 deaths in 2012 [(2)].

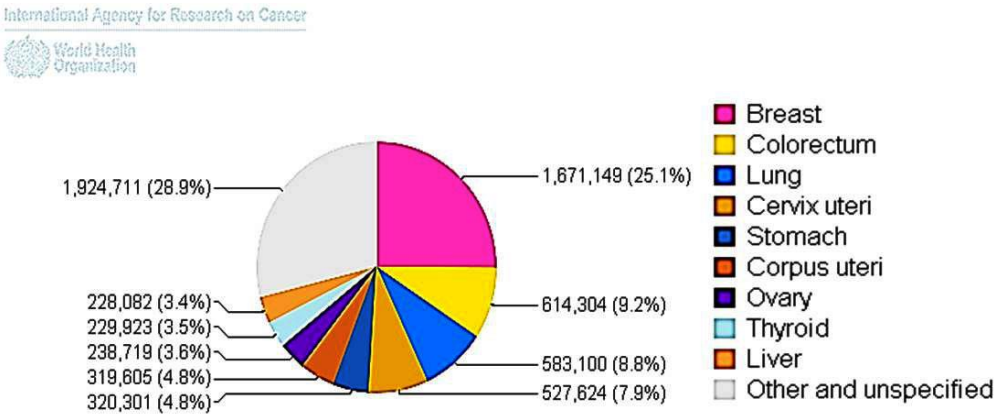

Figure 1 The incidence of breast cancer worldwide

Internet available from: http://globocan.iarc.fr/Pages/fact_sheets_population.aspx

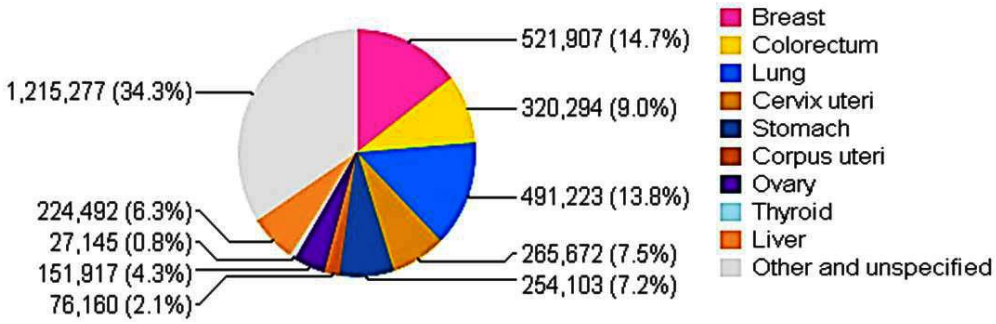

Figure 2 The mortality of breast cancer worldwide

Internet available from: http://globocan.iarc.fr/Pages/fact_sheets_population.aspx

Normal breast

The normal breast is consisting of 15 to 20 lobes and each lobe composed of many lobules with glands that create and store milk. Breast milk from the lobules flows through the ducts to the nipples. Furthermore, the spaces between the lobules and ducts are filled with fibrous tissues and fat and also with the lymph nodes, which are near the breast that connect to axilla (Fig 3)[13].

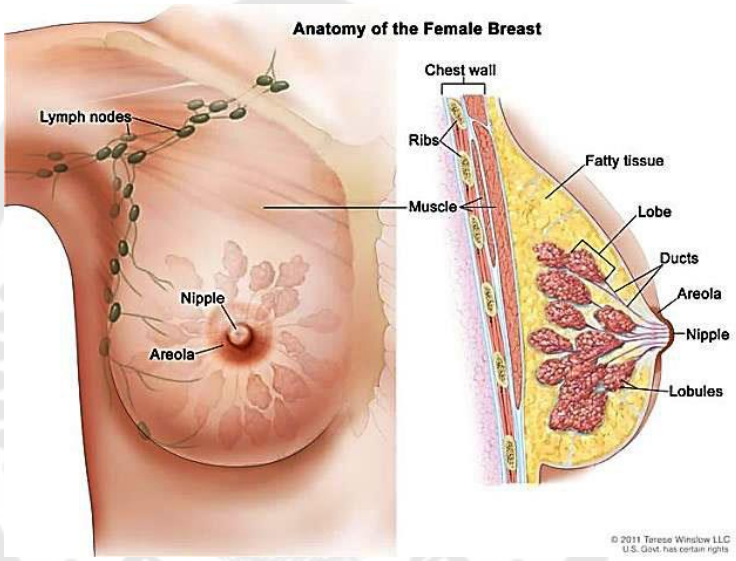

Figure 3 Display of the normal breast

Internet available from: http://m.cancer.gov/images/cdr/live/CDR415520-750.jpg

Definition of breast cancer

Breast cancer is a malignant tumor that starts in the breast cells. This cancer can occur in both the milk ducts and lobules. When the cancer becomes larger, it can spread to surrounding tissues. Furthermore, cancer cells can move from a primary site by invasion into the lymph nodes nearby the breasts.

Types of breast cancer

Breast cancer can be divided into 2 types, which are non-invasive and invasive breast cancers.

1. Non-invasive breast cancer

Non-invasive breast cancers are sometimes called carcinoma in situ ("in place") or pre-cancers. This type is classified in stage 0 and can be divided as 2 subtypes.

1.1 Ductal carcinoma *in situ* (DCIS)

DCIS is the most common type of breast cancer. This cancer occurs in the milk ducts and does not spread into surrounding breast tissue (Fig 4).

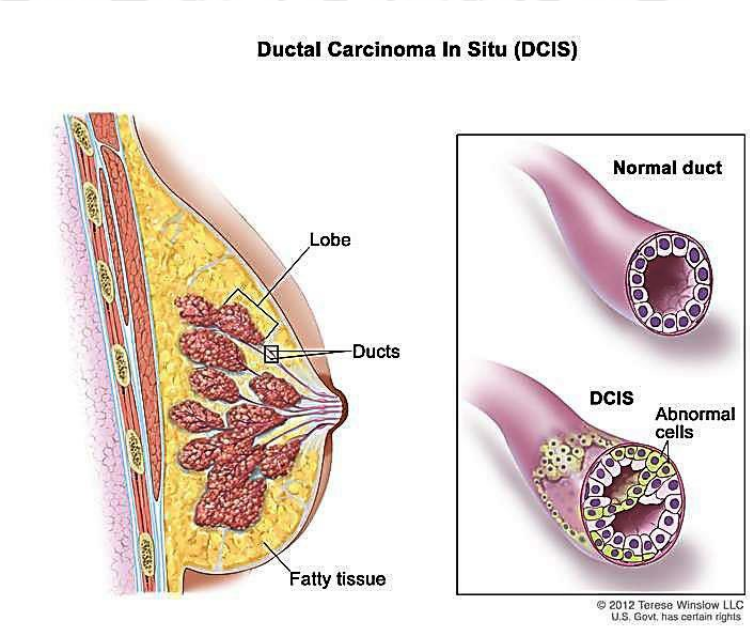

Figure 4 Ductal carcinoma in situ (DCIS)

Internet available from: http://www.cancer.gov/cancertopics/pdq/treatment/breast/Patient/page2

1.2 Lobular carcinoma *in situ* (LCIS)

LCIS is the second most common type of breast cancer. This cancer begins within the lobules of the breast, where milk is produced and secreted through the duct to the nipples (Fig 5).

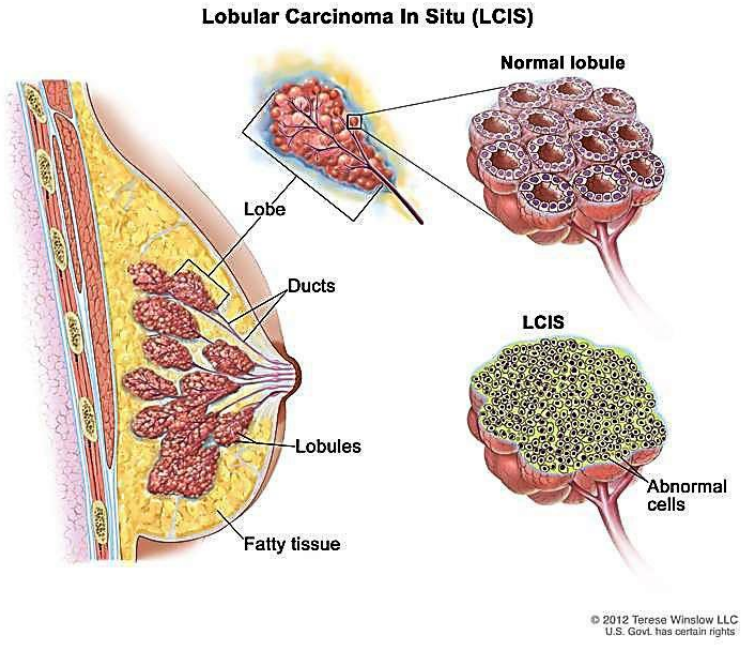

Figure 5 Lobular carcinoma in situ (LCIS)

Internet available from: http://www.cancer.gov/cancertopics/pdq/treatment/breast/Patient/page2

2. Invasive of breast cancer

Invasive breast cancer is a stage that the cancer cells have spread from the primary site (such as milk ducts or lobules) into the surrounding breast tissue and also can spread to the lymph nodes under the arm (axilla) to other parts of the body. Additionally, it also found that this cancer type has a poorer prognosis than DCIS [14].

Stages of breast cancer

The stage of breast cancer can be classified based on the tumor size and how far of cancer cells spreading to the lymph nodes or other parts of the body. It is divided as stage 0, I, II, III and IV.

Stage 0 (carcinoma in situ)

This stage is a pre-cancerous or non-invasive breast cancer. Abnormal cells are found in the lining of the ducts or the lobules of the breast, but it does not spread outside to other tissues in the breast (view in fig 4 and 5).

Stage I (IA, IB)

The stage I is divided into stage IA and IB. In stage IA, the tumor size is no more than 2 centimeters across and has not spread to the lymph nodes. While, in stage IB, the tumor size is in the same range as stage IA with no more than 2 centimeters across, but stage IB can be found spreading into lymph nodes (Fig 6) [15].

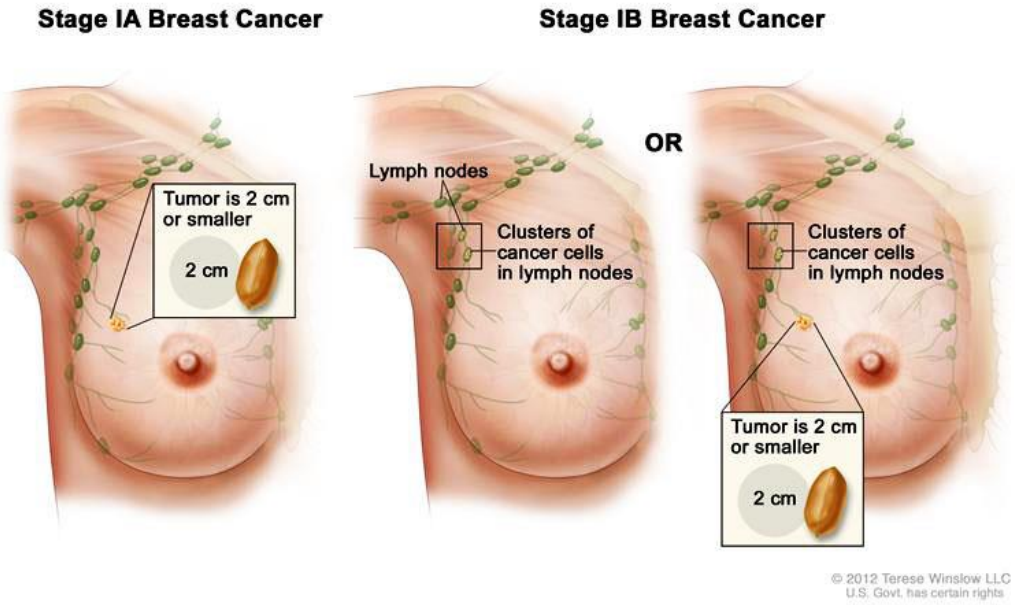

Figure 6 Display breast cancer stage I

Stage II (IIA, IIB)

Stage IIA: in this stage, it can be characterized in two ways. First, the tumor size is no more than 2 centimeters across and cancer cells have spread to underarm lymph nodes or lymph nodes near the breastbone. Second, tumor is between 2 to 5 centimeters across, but it hasn't spread to lymph nodes (Fig 7) [15].

Stage IIB; the tumor in this stage is larger than 2 centimeters but no more than 5 centimeters across, and it has spread to underarm lymph nodes. Or, the tumor is larger than 5 centimeters across, but has not spread to underarm lymph nodes (Fig 8) [15].

Stage III (IIIA, IIIB and IIIC)

Stage IIIA; the tumor size is smaller than 5 centimeters across and spread to 4 to 9 underarm lymph nodes or lymph nodes near the breastbone. Or, the tumor is larger than 5 centimeters across, and found in the lymph nodes, spread 1 to 3 underarm lymph nodes or to the lymph nodes near the breastbone (Fig 9) [15].

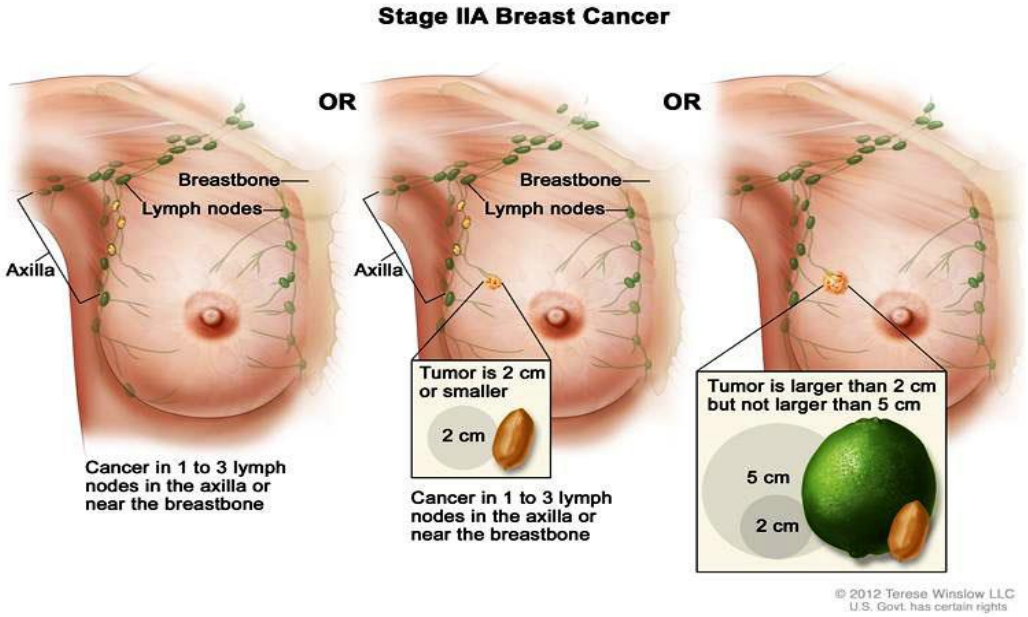

Figure 7 Display stage IIA of breast cancer

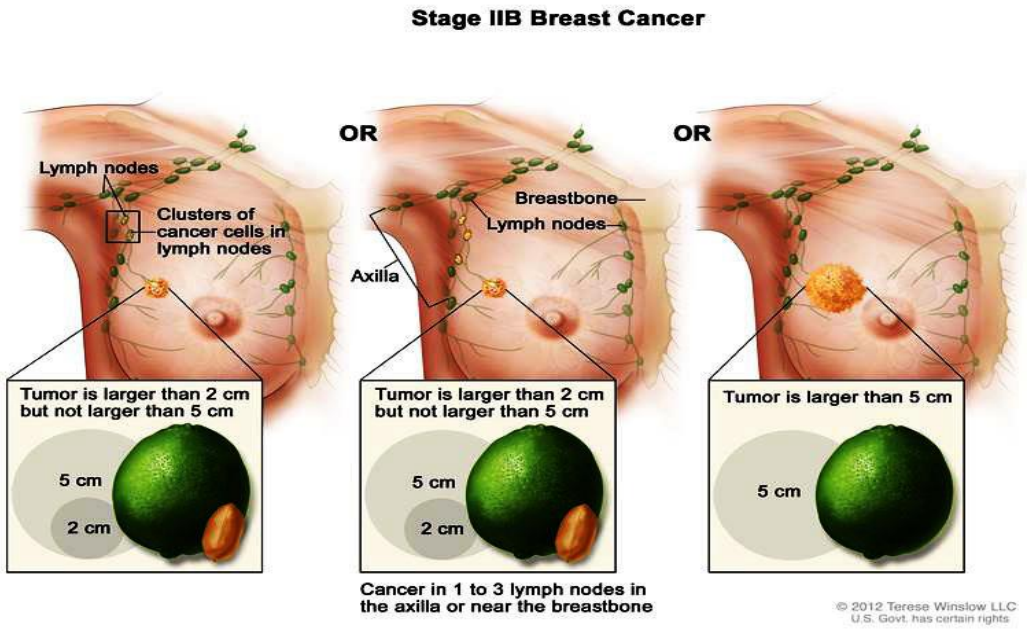

Figure 8 Display stage IIB of breast cancer

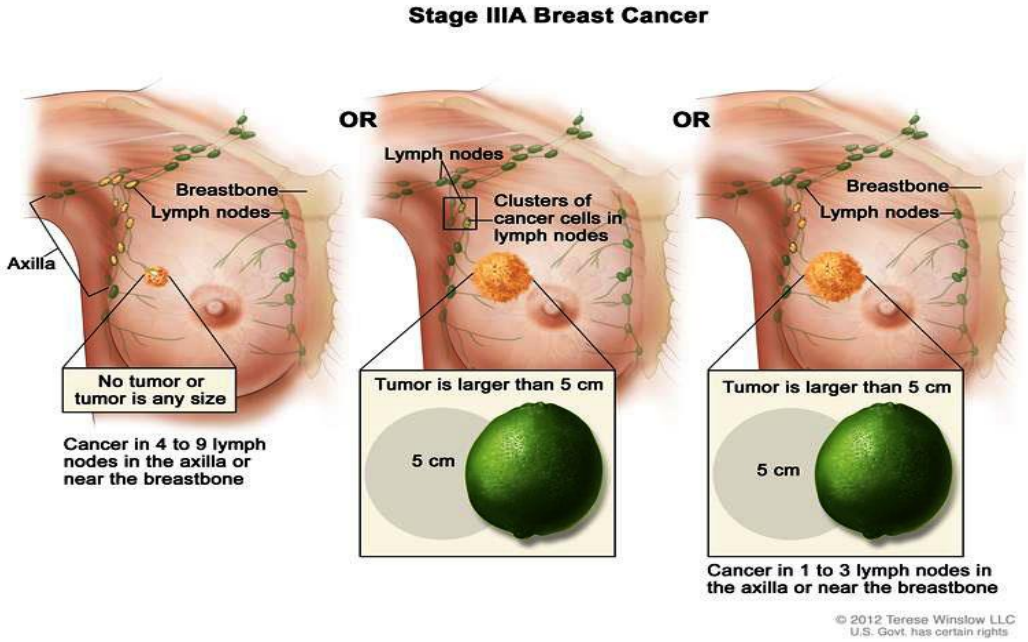

Figure 9 The stage IIIA of breast cancer

Stage IIIB; the tumor can be any size, and it does spread into the chest wall or the skin of the breast. This stage shows inflammatory signs of redness and warmth when the cancer has spread to the skin. The cancer cells block the lymph vessels of the skin which appearance as the dimpled characteristics called “peaud” orange” (like the skin of an orange). In addition, it may spread to underarm lymph nodes or the lymph nodes near the breastbone (Fig 10) [15].

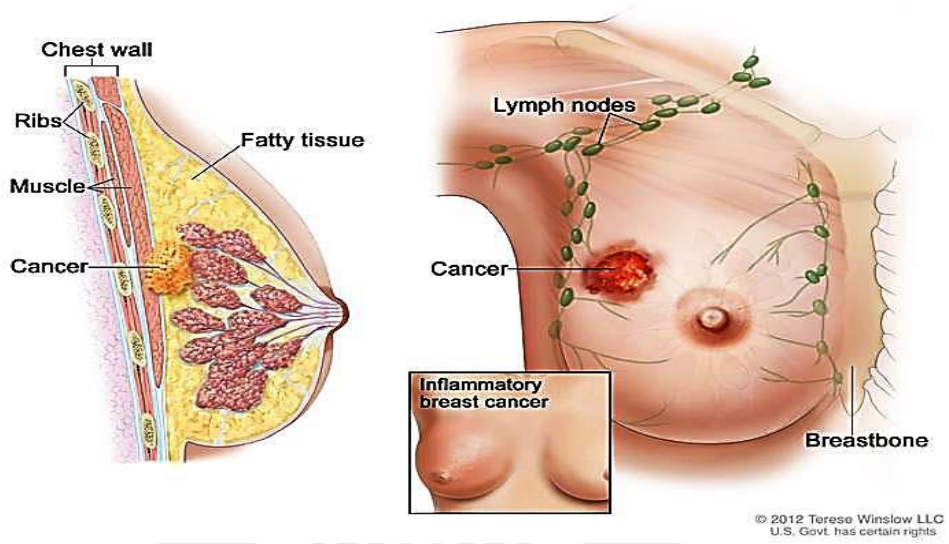

Figure 10 The stage IIIB of breast cancer

Stage IIIC; the tumor can be any size and may spread to breast skin (inflammatory breast cancer) which causes of swelling and ulcer, or spread to lymph nodes behind the breastbone and under the arm, or spread to lymph nodes above or below the collarbone (Fig 11) [15]

Stage IV

The cancer cells spread to other parts of the body, such as lungs, liver, bones or brain. This stage is called secondary or metastatic breast cancer (Fig 12) [15]

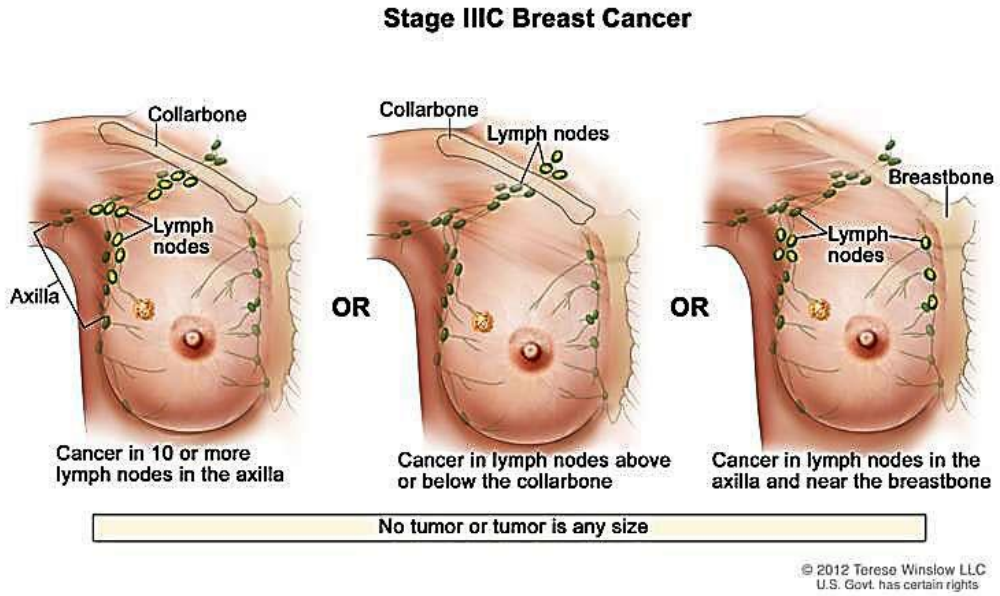

Figure 11 The stage IIIC of breast cancer

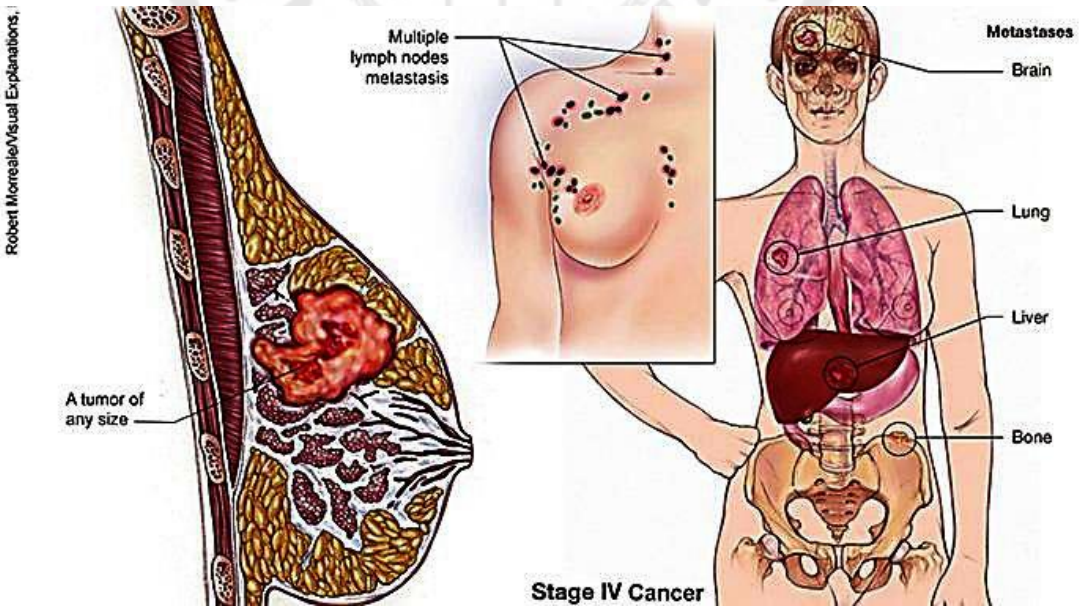

Figure 12 The stage IV of breast cancer.

The signs and symptoms of breast cancer

A new lump or mass in the breast is the first symptom of breast cancer for many women. Sometimes, breast lumps are benign, which means that they are not cancerous. A lump that characterized as cancer is usually painless, hard mass with irregular edges and seldom to present as tender, soft, or rounded. Therefore, the patient who has a lump can be felt painful. Other symptoms can be found such as

- swelling of all or part of the breast
- skin irritation or dimpling
- breast or nipple pain
- nipple retraction (turning inward)
- redness, scaliness, or thickening of the nipple or breast skin
- a nipple discharge other than breast milk [16]

Causes and risk factors

Many factors increase a woman's risk to develop breast cancer.

Higher risk

- being a woman
- age (breast cancer risk increases with age 55 or older)
- family history
- gene mutation (BRCA1 or BRCA2)
- menopause
- menstrual periods (before age 12)
- never having children or having a first child after age 35
- previous chest radiation
- certain benign breast conditions
- using hormone replacement therapy after menopause (HRT)
- exposure to chemicals when food is grilled/prepared
- high breast density on a mammogram

Lower risk

- drinking alcohol
- body weight
- lack of exercise
- smoking
- birth control (oral contraceptives)
- **unhealthy food** [16, 17]

Gene mutation has been reported to be the cause of breast cancer. The most common genes involving with breast cancer are *BRCA1* and *BRCA2* genes, which *BRCA1* mutations are reported as high as 80%. In normal cells, BRCA1 and BRCA2 proteins function as tumor suppressor proteins. They helps in repairing DNA damaged or destroy cells if DNA cannot be repaired. Other genes can also be found to involve with inherited breast cancer, but however not high risk like *BRCA* genes [16]. These genes include:

- *ATM* (ataxia telangiectasia mutated): The *ATM* gene is a serine/threonine protein kinase that functions in DNA repair. The protein is named for the disorder Ataxia telangiectasia caused by mutations of *ATM*. It has been reported that female who was relatives with Ataxia telangiectasia disease linked to increased risk factors breast cancer [18]
- *TP53* (tumor protein p53) gene: The *p53* gene is a tumor suppressor protein which functions in regulating cell division by stopping the rapid growing of abnormal cells. Inherited mutations of this gene cause Li-Fraumeni syndrome and associated with the increasing risk of breast cancer development and several human cancers such as bladder cancer and head and neck squamous cell carcinoma.

However, this cause is found less than 1 percent of all breast cancer cases [19].

- *CHEK2* (Checkpoint Kinase 2) gene: The *CHEK2* gene encodes checkpoint kinase 2 (CHK2) protein. This protein is a tumor suppressor that regulates cell division and also directly phosphorylates and regulates the functions of p53 and BRCA1. The inherited mutations in *CHEK2* gene have been reported to associate with the Li-Fraumeni syndrome and increased about two-fold of breast cancer risk [20].
- *PTEN* (Phosphatase and tensin homolog) gene: The *PTEN* is a tumor suppressor gene that regulates cell cycle by preventing cells from growing and dividing too rapidly. Additionally, this protein has also functioned in cell adhesion, migration and angiogenesis. The mutations of *PTEN* increase the metastatic potential and tumor progression in breast cancer [21].
- *CDH1*: The *CDH1* gene encodes protein cadherin-1 (CAM 120/80 or epithelial cadherin (E-cadherin) or uvomorulin). This protein functions as tumor suppressor that prevents the uncontrolled cells and have also a role in cell adhesion and movement. Inherited mutations of *CDH1* increase a woman's risk of invasive lobular breast cancer [22].
- *STK11*: The *STK1*1 gene encodes a member of the serine/threonine kinase family, which functions as tumor-suppressor protein to regulate apoptosis and cell cycle. The mutation of this gene leads to high risk of several cancers, such as breast, ovary, cervix, lung, testis, pancreas [22, 23].

- *PALB2* (Partner and localizer of BRCA2)*:* The *PALB2* gene makes a protein that interacts with *BRCA2* gene. Mutation of this gene is one important cause of hereditary breast cancer and leads to an increased risk of breast cancer [24].

Breast cancer diagnosis

Many techniques are used to confirm the occurrence of the disease including imaging tests (mammogram, ultrasound and magnetic resonance imaging (MRI), surgical tests (biopsy) (Fig 13) [16], molecular tests (used to determine the appropriate treatment plan to lower the chance of recurrence, consisted of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor *2* (HER2) and Ki67 (related with tumor proliferation)), and blood tests (tumor markers) [16, 25].

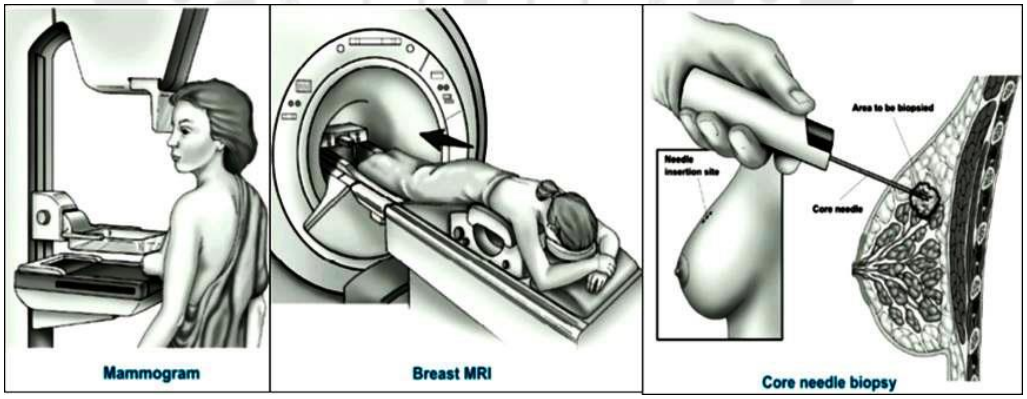

Figure 13 Breast cancer detection by mammogram, MRI and biopsy

Breast cancer classification

After the patients have a biopsy, the breast tissue samples are taken to determine breast cancer type (non-invasive or invasive breast cancer) and grading in

order to select the type of treatment. The grading of breast cancer can be divided into 3 grades are:

- grade 1 is a well differentiated tumor (low grade)
- grade 2 is a moderately differentiated tumor (intermediate grade)
- grade 3 is a poorly differentiated tumor with tend to grow and spread more rapidly (high grade) [26]

In addition, breast cancer cells can be classified according to their receptors in order to offer an appropriate treatment such as chemotherapy, hormone therapy, or target therapy.

1. Classification by hormone receptors and HER2 status

1.1 Hormone receptor-positive

Breast cancer cells contain either estrogen or progesterone receptors, therefore hormone receptor-positive cancers, which are more common in women after menopause, tend to grow slowly and can sometimes reoccur many years after treatment. Breast cancer cells with hormone receptor-positive can be treated by hormone therapy which estrogen receptors are blocked. Hormone therapy drugs are tamoxifen and aromatase inhibitors, anastrozole (Arimidex), letrozole (Femara) or exemestane (Aromasin) [27].

1.2 Hormone receptor-negative

This breast cancer types are not containing either estrogen or progesterone receptors. It is more common in women before menopause which cancer cells tend to grow more quickly than hormone receptor-positive cancers and found that it can be recurred after treatment in the first few years. In addition, breast cancer with hormone receptor-negative can't be treated with hormone therapy drugs [28].

1.3 Human epidermal growth factor (HER2) receptor

HER2 receptor also called ErbB2 is a protein that appears on the surface of some breast cancers. This protein plays an important role in stimulating

growth and survival of breast cancer cells. Breast cancers with HER2 positive are about 15 to 20 percent and 75 percent of HER2 type cancers contain *p53* mutations. This cancer type is fairly poor prognosis and frequently recurrence and metastasis. The drugs for treating breast cancer with HER2 positive are trastuzumab (Herceptin) and lapatinib [4, 29].

1.4 Triple-negative breast cancer

Triple negative breast cancer (TNBC) is a type of breast cancer with the absence of the expression of estrogen receptor (ER-), progesterone receptor (PR-) and human epidermal growth factor (HER-) with approximately 15 percent found of all breast cancers. Epidemiology of TNBC reported that about 170,000 peoples were diagnosed from 1 million cases of breast cancer [30]. TNBC has poorer prognosis, tends to occur in younger women and in women of African-American or Hispanic/Latina and associated with *BRCA1* breast cancer. This cancer type tends to grow, spread quickly or high metastasis, biologically aggressive and does not have hormone receptor targets for treatment. The treatment for TNBC is a combination of surgery, radiation and chemotherapy. The chemotherapy can be divided two parts as neoadjuvant chemotherapy that is given before surgery in order to shrink a tumor and adjuvant chemotherapy which is given after surgery [31]. Some reports suggested that the suitable chemotherapy for TNBC treatment is anthracycline and taxane [32]. It is also reported that TNBC may recur early and spread to other parts of the body, such as liver and has high risk of developing brain metastasis [33].

2. Classification by gene expression

The study on the molecular subtypes made another way to classify breast cancer which is including:

2.1 Luminal A type

The cancer cells that start inside of a duct or gland are called luminal. This type has positive of the estrogen receptor (ER+), so it can be treated by hormonal

therapy. Luminal A cancers then have the best prognosis, low grade and slowly growing. It is found that this type has high in survival rate and low in recurrence rate [34].

2.2 Luminal B type

The luminal B type of breast cancer has the starting point in the inner cells (luminal) lining of the breast such as duct or gland that similar to luminal A. Luminal B cancers are positive for ER and /or PR. In addition, they are highly positive for Ki67 test which means that the cancer cells have a high proliferation rate (growing and dividing) and tend to grow faster than luminal A, poorer prognosis and *p53* mutations are found at about 30 percent [34, 35].

2.3 HER2 type

This breast cancer types have positive of HER2 (HER+) or extra copies of the *HER2* gene and about 75 percent contain *p53* mutations. Breast cancer with HER2 positive tends to grow very fast, worse prognosis, frequent recurrence and metastases. However, the treatment of HER2 type can be treated by target therapy at HER2 with the drug trastuzumab (Herceptin) [36].

2.4 Basal type (basal-like breast cancer)

Breast cancer with basal type has the gene expression patterns similar to basal cells surrounding breast ducts and glands. However, it contains *p53* and *BRCA1* gene mutations that made the cancer cells tend to grow quickly, high grade, poorer prognosis and often aggressive. The current treatment of this type cancer is also like TNBC [37]. The summary of molecular subtype and the biomarker profile can see in table 1 [38].

Table 1 Molecular subtypes and biomarker profile

Molecular subtype	Biomarker profile
Luminal A	ER+ and/or PR+, HER2-, low Ki67
Luminal B	ER+ and/or PR+, HER2+ (or HER2- with high Ki67)
HER2	ER-, PR-, and HER2+
Basal-like	ER-, PR-, HER2-, and/or EGFR+

Treatments for breast cancer

Breast cancer treatment options are varied depending on the stage of cancer and their size, position and how spreading to other parts. Current treatments used for breast cancer include:

- surgery
- radiotherapy
- chemotherapy
- hormonal therapy
- target therapy (also called biological therapies) [39]

The treatment generally for breast cancer is surgery combined with chemotherapy for some breast cancers that do not respond to receptor, or combined with hormone therapy or target therapy for hormone receptor positive cases.

Secondary breast cancer or metastasis

When a tumor becomes larger, it can spread from the primary site to other parts of the body which is called secondary site such as lung, liver or bone. The movement of cancer cells to secondary site is necessary through the bloodstream or lymphatic system. This step called metastasis, which includes migration, adhesion and invasion (Fig 14). The process of cancer cell metastasis is composed of several steps which are:

1) Detachment of cancer cells from the primary site

2) Invasion into extracellular matrix (ECM)

3) Intravasation into blood or lymphatic vessels

4) Dissemination in the bloodstream or the lymphatic system

5) Extravasation, form micrometastases, survival, perhaps dormancy and outgrowth at a secondary site [40]

Each step requires many specific molecular interactions that make contribution for cancer cell movement in surrounding ECM. These interactions are mediated by contact between cells and ECM, by direct cell-cell contact, and by secreted factors.

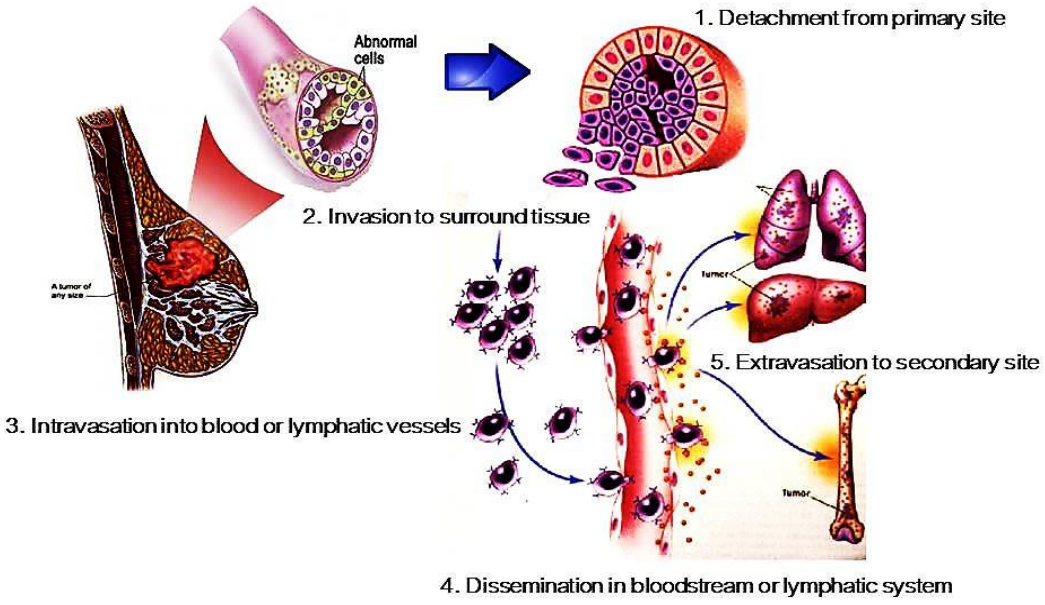

Figure 14 The metastatic cascade

Regulation of cell migration and invasion

Cell migration is essential for many biological processes such as embryonic morphogenesis, immune surveillance, and wound repair and regeneration. Error regulations of cell migration are the cause of many diseases including vascular disease, tumor formation and metastasis [41]. Therefore, understanding of the regulation of cell migration is important for controlling the diseases and leading to effective therapeutic.

Cell migration is initiated by the extension of the cell membrane, or can be described as protrusion at the leading edge in the direction of movement. To initiate migration process, this leading edge is adhered to the surface during cell moving, while the trailing edge is de-adhered. Other force helps in the last step is the pulling of the whole cell body forward by contractile forces of the cell body and rear (Fig 15) [42] The structures of protrusion formed by migrating and invading cells, can be classified based on morphological, structural, and functional characters. The cells with filopodia and lamellipodia (so-called two-dimensional structures (2D) are cells that begin to migrate which can be observed by membrane protrusion at the leading edge. Meanwhile, during migration, cells will use invadopodia and podosome (so-called three-dimensional structure (3D) with membrane protrusions at the invading front to enter (invade) the dense rigid ECM. Both invadopodia and podosome have a specific function in ECM remodeling by using the activity of matrix metalloproteinase (MMP) enzymes [43] (Fig. 16) [41].

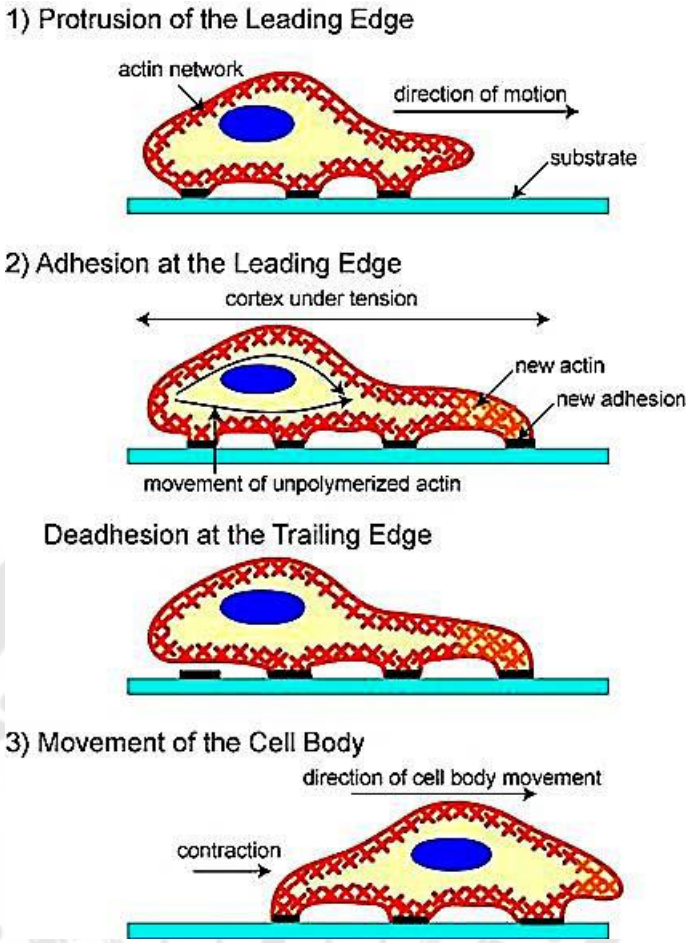

Figure 15 The stage of cell movement

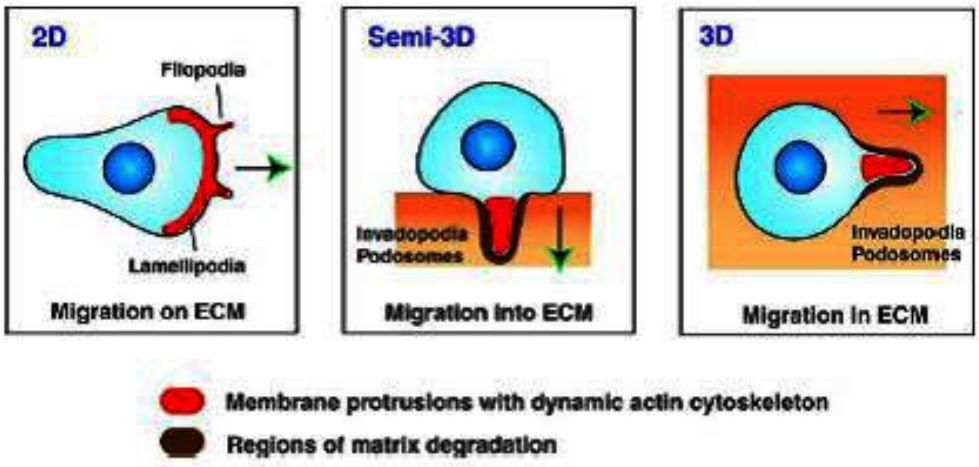

Figure 16 The different environments of cell migration and membrane protrusions

The driving force of cell migration, invasion and essential molecules

1. Lamellipodia

Lamellipodia are characterized as a flat, sheet-like membrane protrusions formed at the leading edge of a migrating cell. This structure has a major role in driving cell migration by attaching to the substrate on ECM surface and producing force to pull the cell body forward during the process of cell migration. This procedure made the cancer cells crawl on ECM towards blood /or lymph vessels from the primary tumor. The driving force of lamellipodia is generated by actin polymerization which requires generation of free barbed ends of actin filaments at the leading edge [35, 36].

1.1 Essential molecules for regulation of lamellipodia formation

1.1.1 WASP family and Arp2/3 complex

Regulation of lamellipodia formation is involved with several mediators and required a complex cascade. The crucial factor is the process of actin polymerization and depolymerization at the cytosolic part, especially the protrusion end. At this site, the intracellular signaling starts from the generation of actin filament barbed ends which oriented toward the membrane [37]. This barbed ends are further activated by Arp2/3 complex and WASP (Wiskott–Aldrich Syndrome protein/Scar family proteins) to promote the nucleation of a branched and elongated of actin filament network. However, when the cells are moving forward, the filament of actin has to be in the dynamic stage by switching between polymerization and depolymerization along the way of movement. (Fig 17) Therefore, it is required some mediators to intitate the depolymerization of actin [38]. To this aim, the actin depolymerization factor (ADF)/cofilin activates the depolymerization at minus end of actin filaments and producing ADP-actin [44, 45]. These actin monomers can then be recycled back in the form of network filaments by the activation of profilin that control the exchange of ADP to ATP. The level of expression of these mediators is then important for cancer cells movement and enhancing of the metastatic property. For example, the overexpression of cofilin was related to the metastasis ability in breast, lung and glioblastoma cancers [46, 47]

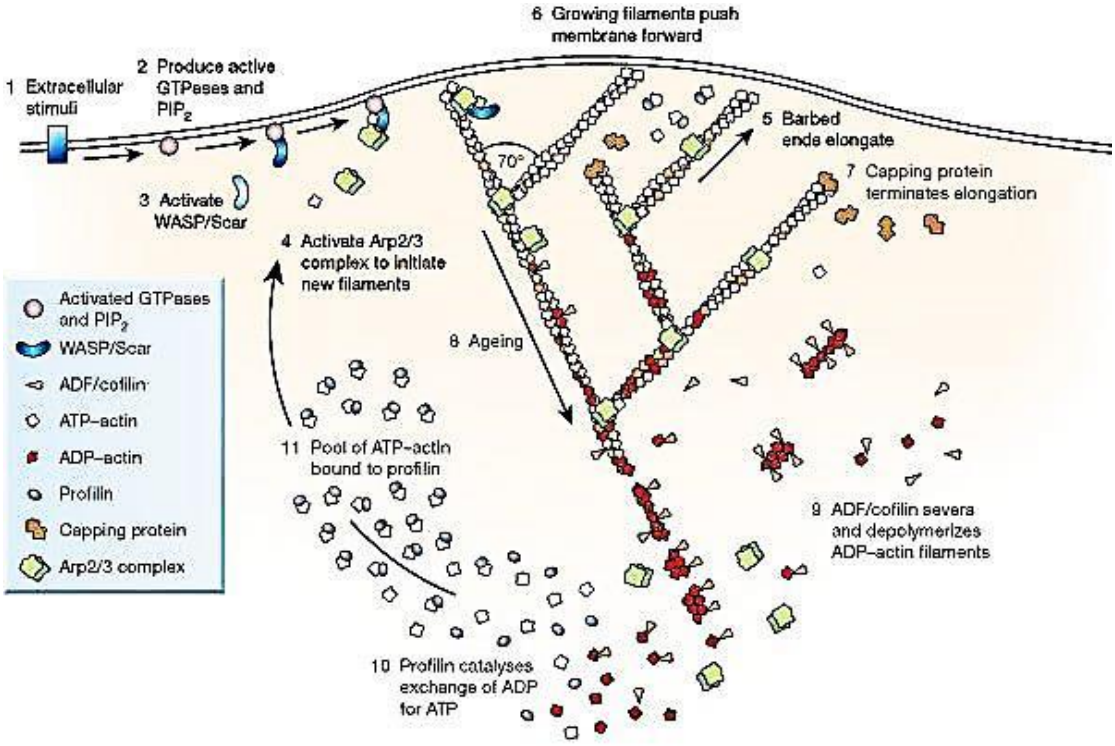

Figure 17 The actin dynamics at the leading edge of motile cells

Internet available from: The dendritic-nucleation model for protrusion of lamellipodia (http://www.nature.com/nature/journal/v422/n6933/fig_tab/nature01598_F1.html)

1.1.2 Rho family GTPase

Rho family GTPases that having a prominent role in the driving of lamellipodia are Rac and Cdc42 (Cell division control protein 42 homolog) [48, 49]. Rac has a function in regulating the formation of lamellipodium and membrane ruffling, while Cdc42 regulates filopodium formation [49]. Rac regulates the function of WAVEs in lamellipodium and membrane ruffle formation. Rac comprises of Rac1, Rac2 and Rac3. Previously reported that the cells cannot migrate when Rac is inhibited [50, 51]. Rac1 (Ras-related C3 botulinum toxin substrate 1) is a member of the Rac subfamily with a small (~21 kDa) signaling G protein and ubiquitously expressed. The functions of Rac1 are controlling of cell growth, cell motility by regulate the formation of lamellipodia, and

the activation of protein kinases [52]. Rac1 mutations are resulting in metastasis of cancer such as liver, melanoma cancer as well as in human breast cancer [44, 53, 54]. Rac2 (Ras-related C3 botulinum toxin substrate 2) is a small (~21 kDa) signaling G protein that mainly function as the activator of NADPH oxidase and consequently generation of reactive oxygen species (ROS) in hematopoietic cells [55, 56]. Rac3 (Ras-related C3 botulinum toxin substrate 3) is a member of the Rac subfamily of the Rho family of small G proteins which has highly expressed in brain, and upregulated in some breast cancer cell lines [57]. Then, only Rac1 is crucial for the regulation of cell migration by controlling of leamellipodia formation. Upstream signaling of Rac1 has been shown to mediate through the tyrosine kinases and G-protein-coupled receptors which depending on phosphoinositol3-kinase (PI3-K) activity [50, 51]. The activation of Rac leads to WAVEs stimulation that affects Arp2/3 complex for branching actin filaments [55, 62].

2. Invadopodia

Invadopodia or invasive feet are actin rich protrusive structure that capable to extend into the ECM. This structure contains matrix degradation enzymes or matrix metalloproteinases (MMPs) with a function of ECM degradation. These enzymes are frequently found in the metastatic process of cancer cells that invading to tissue surrounding [58, 59].

2.1 Essential molecules for regulation of invadopodia formation (N-WASP and cortactin)

The essential components in the structure of invadopodia are N-WASP and cortactin (Fig 18) [60]. These proteins have been reported to be upregulated in malignant cancer cell. N-WASP (neural WASP) is a member of WASP family that interacts with upstream regulators such as Cdc42 (for filopodia formation), SH3 domain containing proteins (Nck and Grb2) and phosphoinositides. N-WASP is phosphorylated through Src family kinases and signaling pathway of N-WASP is identified as necessary for the invadopodia formation in cancer cells by inducing actin filament nucleation through activation of Arp2/3 complex which leading to promote cancer cell invasion and

metastasis [53, 54]. Additionally, N-WASP has been identified as a key regulator in the formation of matrix-degrading structures of invadopodia. Also, knockdown of N-WASP suppressed the invadopodia formation and matrix degrading activity in MTLn3 cells [55, 56].

Cortactin is a protein that is located in cytoplasm and activated by phosphorylation of tyrosine kinases (Src kinase) to promote polymerization and rearrangement of the actin cytoskeleton [57]. Cortactin has been shown to bind and cross link with actin filaments and also directly activates Arp2/3 complex to promote nucleation of actin. In addition, when the cortactin binds to N-WASP which leading to indirectly promote Arp2/3 complex and activating it. In addition, Arp2/3 complex has been shown to have a relationship with vinculin at adhesion sites and may be possible that cortactin is involved in actin assembly at adhesion site [61, 62]. However, the protrusive force of lamellipodia does not require cortactin but essential for suitable barbed end formation and assembly of new adhesions for persistence of cell migration [63]. Previously reported in breast cancer cells found that cortactin localizes at invadopodia and inhibition of cortactin indicates that the invasive activity of breast cancer cells was blocked [64].

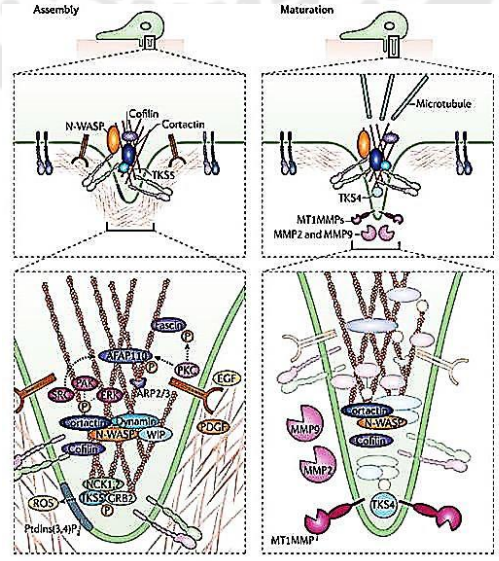

Figure 18 Invadopodium formation and essential components

Cell adhesion

Cell adhesion is a crucial step in normal cells, viral binding to host cells, bacteria colonization and regulates growth and cancer metastasis. Cell adhesion is the binding of a cell to the surface or substrate on ECM called cell-matrix adhesion. This process is a key regulator of cell migration by forming focal adhesion complex and migration through ECM matrix [65].

1. Cell-matrix adhesion

The extracellular matrix (ECM) is a cluster of extracellular molecules that providing cellular support in all tissues and organs. ECM is composed of matrix molecules such as fibronectin, collagens, laminins, proteoglycans, and non-matrix proteins including growth factors [66]. The cell is required to adhere with the ECM to mediate direct interactions of the cell with its extracellular environment. Cell-matrix adhesions are important for cell migration, tissue organization, and differentiation. Additionally, the signals of matrix adhesion are cooperating with other pathways that control biological processes such as cell survival, cell proliferation, wound healing, and tumorigenesis [67].

1.1 The essential components of cell-matrix adhesions

1) Cell adhesion molecules (CAMs)

The proteins located on the cell surface are called cell adhesion molecules (CAMs) which have a function in binding with other cells or binding cells to ECM. CAMs are transmembrane receptors containing three domains including intracellular domain that interacts with the cytoskeleton, a transmembrane domain and extracellular domain that interacts with other CAMs [68]. This protein plays a critical role in regulating signal transduction of cells, such as cell cycle progression, adhesion, inflammatory response, cell migration as well as in cancer cells found that CAMs plays a key role in regulation of cell invasion and metastasis [69]. CAMs are composed of four protein families including Ig (immunoglobulin) superfamily (IgSF CAMs) which has a function in immune system by binding to a foreign molecule in the body, integrin which

is responsible for mediation of the interactions of cells and ECM , cadherins which have the role in intermediate adherin junctions that link to actin filament network, and selectins which are found in white blood cells and play a key role in inflammatory response and mediating the invasion of neutrophils from the bloodstream into the area of injury [70, 71]. The structure of CAMs families are shown in figure 19 [72]. According to the above mentioned, integrins have a major role in cell surface adhesion by mediating cell-matrix adhesion and leading to various signaling pathways [73].

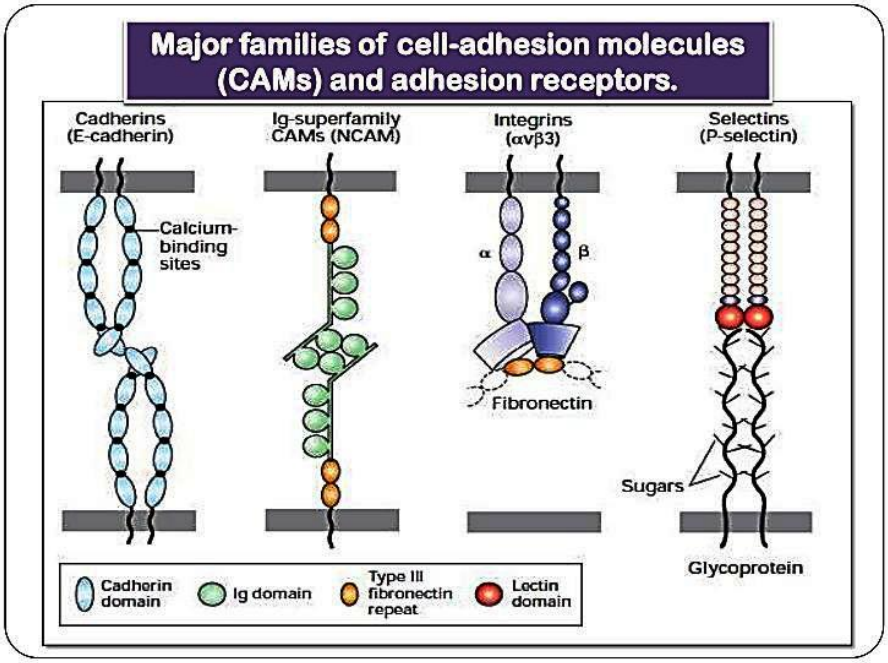

Figure 19 The structure of CAMs families

Internet available from: http://image.slidesharecdn.com/adhesionmolecules-111109065715 php app01/95/adhesion-molecules-2-728.jpg?cb=1400781811

1.1) Integrins

Integrins are transmembrane receptors and play a major role in cell adhesion by crosslink with cell-cell and cell-ECM matrix interactions followed by trigger

signal transduction. This resulting in activation of transcription factors involved regulation of cell cycle, cell survival, cell shape and motility. In cancer cells, integrins are strongly associated to signaling pathways for stimulation of cancer growth, progression and metastasis. Moreover, it also found that integrins are implicated in angiogenesis [73, 74].

1.2) Structure of integrins

Integrins comprised of α- and β- chains heterocomplexes and act as integral membrane receptors. They form focal adhesion contacts with various ligands in ECM including fibronectin, laminin, vitronectin, collagens, tenascin and fibrinogen. In addition, integrins have been identified as 18 α subunits and 8 β subunits and formed into 24 distinct integrins [75]. (Fig 20)

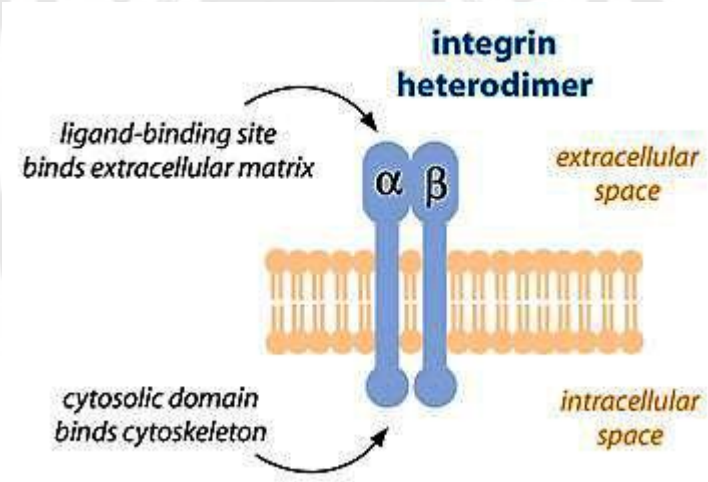

Figure 20 The structure of integrin

Internet available from: http://www.scq.ubc.ca/the-role-of-integrins-in-wound-healing/

1.3) Activation of integrins

Integrins modulate adhesion through a conformational change induced by talin, which is an adaptor protein that binds to β tail subunit of integrins. As a

result, integrin receptors are activated providing the connection with actin cytoskeleton. Adaptor proteins not only talin but also vinculin and α-actinin that are important for adhesion process. Vinculin does not bind integrins directly, but it binds with talin and acts as a crosslinker between the talin–actin interactions. It recruited at focal adhesion and also binds to Arp2/3 complex which nucleates actin filaments in the lamellipodium [76-78]. There is also paxillin, a signal transduction adaptor protein that binds to β tail of integrins [79]. The roles of paxillin are targeted to vinculin and recruitment of protein tyrosine kinases such as Src and focal adhesion kinase (FAK). The phosphorylation of paxillin is initiated by Src and FAK, which affects to p130 Crk-associated substrate (p130Cas). activated as a results Rac stimulated that leads to cell spreading and motility [80, 81]

2. Focal adhesions (FAs)

2.1 Focal adhesions (FAs) structure

Integrins activation by attachment of cell to ECM components lead to recruitment of essential proteins to binding sites, consequently the focal adhesions (FAs) are formed and stimulate signaling pathways that involve cell migration and invasion. FAs are sites where adhesion is mediated by integrins to connect with the actin cytoskeleton. Also, several of adaptor proteins such as talin, vinculin, α-actinin and paxillin provide strong linkages of actin cytoskeleton, firmly to connect with ECM and recruit protein kinases such as FAK, Src as a result stimulated signaling pathways (Fig 19) [72, 82]. Therefore, FAs are proposed as an important mechanism for regulating cell adhesion, migration and invasion with mainly a function of FAK and Src [83, 84]

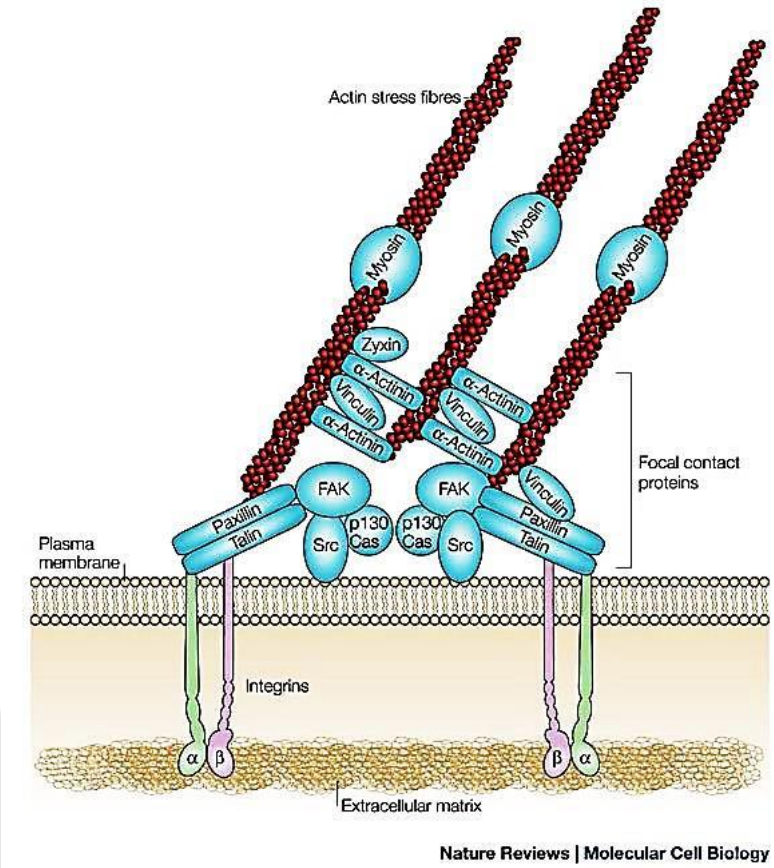

Figure 21 Focal adhesions and essential components

2.2 Regulations of focal adhesion (FA)

2.2.1 FAs assembly

The adhesion of cells to the ECM through integrins, lead to the formation of FA complexes which recruiting essential proteins as discussed above. The initial step of FA assembly is talin that binds to β subunit of integrin. Subsequently, integrins were clustered. The activation of integrins at actin filaments affects the recruitment of necessary adaptor proteins and protein kinases for FA formation. There are also recruited zyxin and tensin to FA with the function is remodeling into FA and stabilize the protrusion. The small GTPase, Rac controls the actin dynamics that contribution to forward protrusion (Fig 22) [(85)].

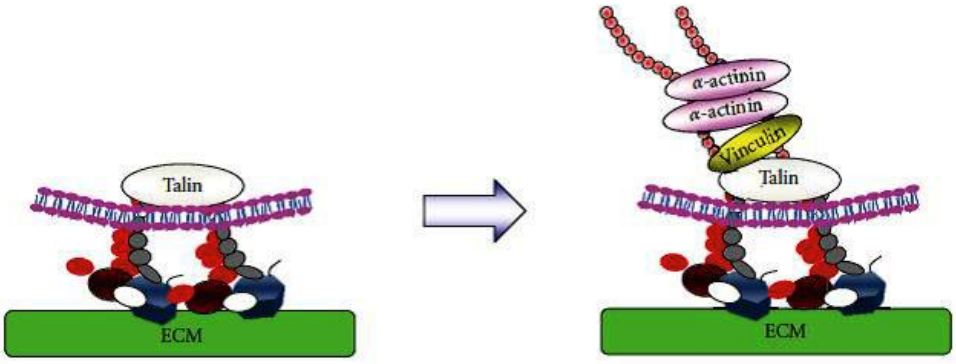

Figure 22 The initiation of FA assembly by talin induced integrins clustering

2.2.2 FAs disassembly

The disassembly of FAs is the process that occurred when the cell is required to increase cell migration via the reduction of FAs and cell adhesion [78]. FAs disassembly comprised of many factors which are microtubules (MTs), dynamins, Growth factor receptor-bound protein 2 (Grb2), FAK, Src and calpain. Microtubules (MTs) play an important role in inducing FA disassembly by triggering the release of cell adhesion through the extension of itself to FAs. In addition, there are also Rho family GTPases that regulates the extension of MTs and stimulate cell migration [86]. The previous studies reported that the disruption of MTs has resulted in focal adhesion formation [87]. After the MTs are triggered by FA disassembly, Src phosphorylation is occurred at Tyr ^{397} and multiple tyrosine residues within FAK involved Tyr^{925} which is necessary for the interaction between FAK and Grb2 [88]. The binding of FAK and Grb2 are recruited dynamin to FAs. These complexes (FAK, Grb2 and dynamin) are responsible for integrins internalization and induced FAs disassembly [89] (Fig 23) [90].

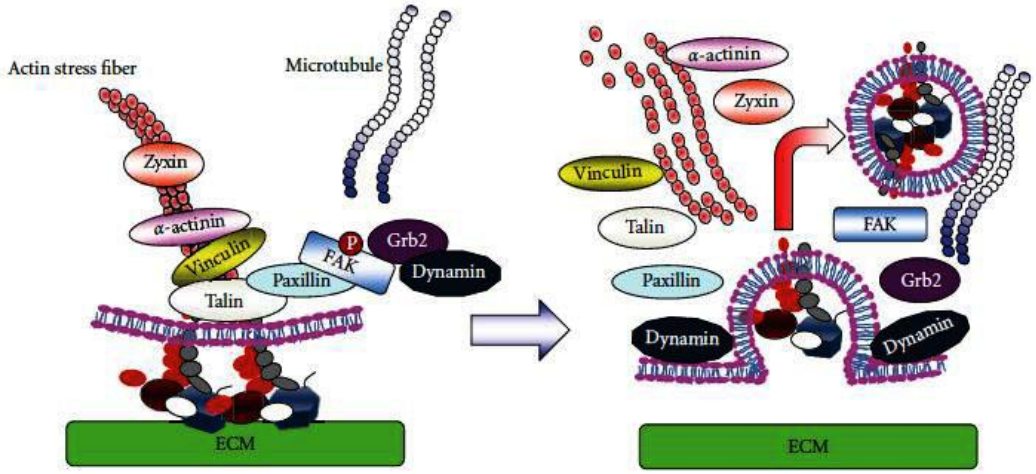

Figure 23 The disassembly of FAs

Moreover, calpain, a family of intracellular calcium-dependent (proteolytic enzymes), have been reported to regulate cell migration by modulating adhesion dynamics, cytoskeletal remodeling, cell differentiation and apoptosis [91, 92]. Calpain was induced by the interaction between Endoplasmic reticulum (ER) Ca^{2+} sensor STIM1 and plasma membrane, SOC channel Orai1 leading to Ca^{2+} influx and promotes calpain-mediated disassembly by cleaving the proteins localized to FA such as talin, vinculin, paxillin and FAK (Figure 24) [93]. Therefore, the disassembly of FAs occurred all the time when the cell is in the process of migration and this process switched with FAs assembly of cell adhesion. In breast cancer cells, it has been reported that inhibition of FA disassembly associated with the inhibition of cell invasion and metastasis [94].

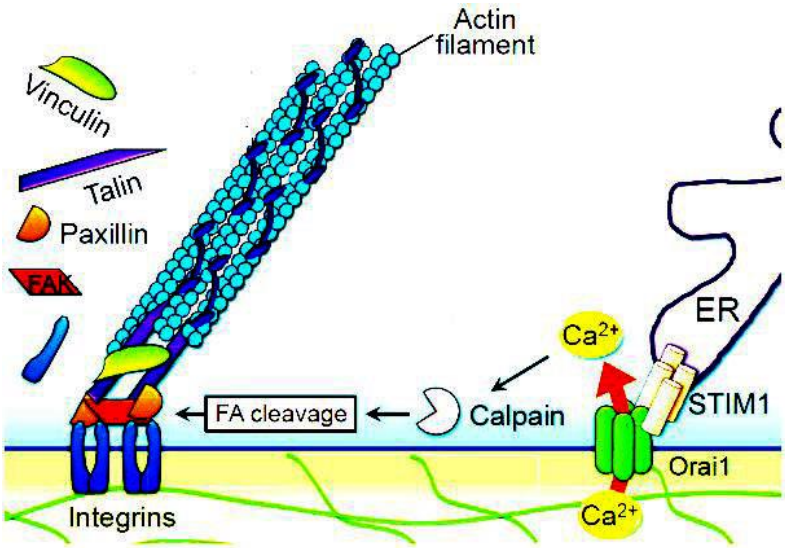

Figure 24 Calpain regulates FA disassembly

3. Focal adhesion kinase (FAK)

Focal adhesion kinase (FAK), also known as protein tyrosine kinase 2 (PTK2), which is a protein involved in cellular adhesion and spreading mechanism, and plays an important role in integrins mediated signal transduction [84]. It has been reported that FAK was blocked in breast cancer cells leading to less metastasis [95]. FAK was also found to be overexpressed in colorectal carcinoma and liver metastases [96]. In addition, FAK associated with angiogenesis both in normal cells (human umbilical vein endothelial cells (HUVEC) and in cancer cells by modulated vascular endothelial growth factor receptors (VEGFRs) [97, 98]. Therefore, FAK is a critical factor to mediate signal transduction that associated with integrins, especially in cancer cells progression, metastasis and angiogenesis. Hence, FAK is a new target therapy for many cancers [99].

3.1 The structure of FAK

FAK contains a N-terminal FERM (protein 4.1, ezrin, radixin and moesin homology) domain, kinase domain, and C-terminal of focal adhesion targeting (FAT)

domain (Fig 25) [84]. FERM domain mediates FAK interactions with the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, the ETK tyrosine kinase and ezrin-radixin-moesin (ERM) family of proteins. In addition, FAK interact with signaling proteins such as the JAK family of tyrosine kinases and several tyrosine phosphatases and the FERM domain can be conjugated to SUMO (small ubiquitin-related modifier) at Lys152 with the functions as nuclear transport, transcriptional regulation, apoptosis, and protein stability [88]. The signaling pathway of talin FERM domain binds to β subunit of integrins could induce the interaction of Janus kinase (JAK), a non-receptor tyrosine kinase that transduces cytokine-mediated signals via the JAK-STAT pathway that plays a role in cellular processes such as proliferation, apoptosis and migration [100, 101]. Additionally, ERM proteins bind through FERM domains to CD44 which is a cell-surface glycoprotein that mediates cell-cell interaction, cell adhesion, migration and cooperates with MMPs to regulate cell invasion [102, 103]. FAT domain contains three proline-rich (PRR1–3) sequences as docking sites for Src homology-3 (SH3) domain-containing proteins including p130cas with a function of recruitment additional signalling proteins, the GTPase regulator associated with FAK (GRAF) and the Arf-GTPase-activating protein ASAP1 [104]. In addition to FAT domain, it is also recruited FAK to focal contacts by interaction with integrin-associated proteins such as talin and paxillin. This complex proteins link to stimulate Rho GTPases by binding to guanine nucleotide-exchange factors (GEFs) such as p190 RhoGEF. FAK is activated through autophosphorylation at tyrosine 397 (Tyr^{397}) and binds Src on multiple tyrosine residues including Tyr^{576}, Tyr^{577} (within kinase domain), Tyr^{861}(locate between kinase and FAT domain) and Tyr^{925} within FAT domain. Phosphorylation on Tyr^{397} creates binding site of Src-homology-2 (SH2) for Src, phospholipase Cγ (PLCγ), suppressor of cytokine signalling (SOCS), growth-factor-receptor bound protein 7 (GRB7), the Shc adaptor protein, p120 RasGAP and the p85 subunit of phosphatidylinositol 3-kinase (PI3-K). Furthermore, phosphorylated Tyr^{861} leads to mediate FAK interaction with talin and paxillin while phosphorylation at Tyr^{925} is essential for FAK interact with Grb2 that allows recruitment of dynamin to FAs [105]. In cancer cells, it has been reported that FAK

autophosphorylation at Tyr^{397} increased cancer cells migration and invasion. Overexpression of FAK is found in a broad range in human cancer, such as breast, colon, thyroid, prostate, oral cavity, liver, stomach, skin, head and neck, lung, kidney, pancreas, bone and ovary [95, 96, 106].

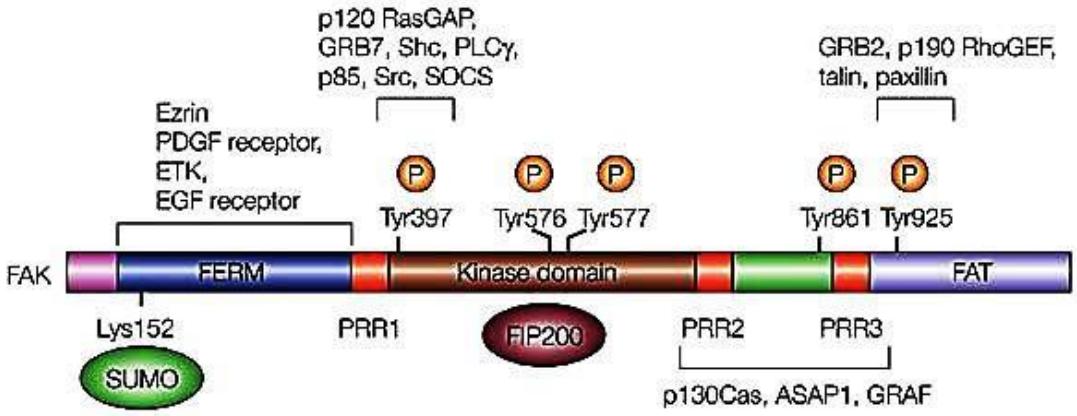

Figure 25 The structure of FAK

3.2 Signaling pathway of FAK to control cell adhesion, migration and invasion

The interaction between cell-matrix adhesion is induced by the clustering of integrins which further recruitment of necessary proteins for FA formation such as talin, vinculin, paxillin and FAK. The autophosphorylation of FAK at Tyr^{397} causes FAK activation which further phosphorylates PI3-K. This PI3-K directly activates RAC1 leading to stimulation WAVEs and induce Arp2/3 complex for branching actin filament which then promotes cell migration and invasion [95]. Previous studies have reported that FAK, PI3-K and RAC1 are overexpressed in many cancers such as breast, colon, lung and liver cancer and affect to increase of cancer metastasis. Moreover, FAK induces the expression of MMP2 and MMP9 for ECM degradation in order to invade to surrounding tissue [107-109]. The association of FAK with PI3-K increases the stimulation of

vascular endothelial growth factor receptor-2 (VEGFR-2) which then binding to VEGF-A promote migration and angiogenesis of endothelial cells (ECs) [99, 110]

3.3 FAK related to mitogen-activated protein kinase (MAPK)

MAPKs are important regulators in cellular response such as stress, heat shock, proinflammatory cytokines and regulate cellular functions of cell proliferation, gene expression, differentiation, motility, cell survival and apoptosis [111, 112]. MAPK family in mammalian are extracellular signal regulate kinase (ERK), p38 and c-Jun N-terminal kinase (JNK, also known as stress-activated protein kinase or SAPK). In each MAPKs signaling pathway, it usually includes at least three components of the MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK that activate in cascade [113, 114] (Fig 26).

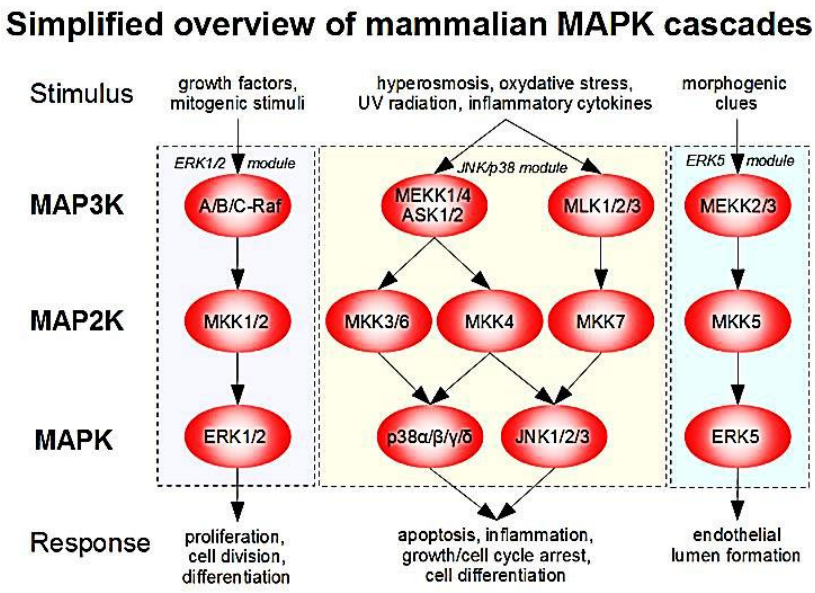

Figure 26 The mammalian MAPK cascade

Internet available from: https://commons.wikimedia.org/wiki/File:MAPK-pathway-mammalian.png

Previously reported that, MAPK activation in cancer cells leading to the stimulation of cell proliferation, evasion of apoptosis, increasing of cell invasion, adhesion, cancer metastasis, and angiogenesis [104, 115]. MAPK has been reported that it is a downstream target of FAK which resulting in cell proliferation and survival [116]. Additionally, FAK has been shown to related with ERK signaling pathway to induce cancer growth [117]. Interestingly, FAK downregulation followed by inhibiting ERK phosphorylation is shown to reduce MDA-B231 adhesion to fibronectin and vitronectin, and reduced mRNA expression of integrin receptor $\alpha 5\beta 1$ and $\alpha v\beta 3$ [118, 119]. In addition, JNK plays an important role in the cellular apoptosis pathway [120] and JNK has also been shown the significant on cancer progression and development [121]. The inhibition of FAK/JNK signaling leads to the inhibition of cell migration, invasion and reduced MMP-2 and -9 expression [122]. Also, the function of P38 MAPK are responsible to stress such as cytokines, ultraviolet irradiation and involving in cell cycle, apoptosis and autophagy, development, cell differentiation, senescence and tumorigenesis in specific cell types [123]. Moreover, P38 MAPK, and JNK have been reported to regulate the balance of autophagy and apoptosis [124, 125].

Phosphatidylinositol 3 – kinases (PI3-Ks)

PI3-K is a family of intracellular lipid kinases that are important for cell proliferation, survival, cytoskeleton reorganization and vesicle trafficking [126]. PI3-Ks are divided into three different classes base on structure and function. Class I PI3-K family are liable for phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2), and phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3) production and activated by G protein-coupled receptors and tyrosine kinase receptors. Class I PI3-K is heterodimeric molecules comprised of a regulatory and catalytic subunits which further divided into two subsets as class IA PI3-K which is

comprised of p110 catalytic subunit with three variants: p110α, p110β and p110δ, and p85 regulatory subunit with five variants: p85α, p55α, p50α, p85β and p55γ [127]. It has been reported that p110α and p110β are commonly found in all cells, but p110δ is expressed generally in leukocytes [111]. The p85 regulartory subunit binds to p110 catalytic subunit and recruits the heterodimer of p85 and p110 to its substrate phosphatidylinositol 4,5-bisphosphate, which effects on Akt activation, which known as a downstream effector of PI3-K signaling [112]. It has been reported that the catalytic subunit p110α is frequently found mutation in most of human cancer [113]. Class IB PI3-K is comprised of the regulatory p101 and catalytic p110γ subunits. Class II and class III PI3-K are distinguished from the class I both in a function and structure as class II has three catalytic isoforms but have no regulatory subunit and unlike to class I which function as catalyse the production of PI(3)P from PI and PI(3,4)P2 from PIP, while class III produced only PI(3)P from PI and involved in protein trafficking and vesicle [114]

PI3-K dependent AKT signaling pathway

Protein kinase B (PKB), also known as AKT, is a serine/threonine-specific protein kinase. AKT plays an important role in intracellular processes such as cell proliferation, apoptosis and migration [128]. AKT is the downstream target of PI3-K signaling pathway and it can then be phosphorylated by its activating kinases [129]. The signaling of PI3K/AKT is modulated in human cancer both in cancer progression and resistance to chemotherapy. PI3K/AKT pathway activation was shown to inhibit apoptosis and increase cell growth and proliferation [130] by the generation of PIP3 at the plasma membrane which then recruits pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1) and activates Akt. The levels of PIP3 are tightly regulated by tumor suppressor PTEN (phosphatase and tensin homology) activity. The function of PTEN is to convert PIP3 back to phosphatidylinositol 4,5-bisphosphate, which the effect is opposed to PI3-K. Thus, PTEN is an inhibitor of PI3-K/Akt signaling [116, 117]. However once Akt is activated, Akt further activates mouse double minute 2 homolog (MDM-2) to

inhibit p53 and thus stop apoptosis. Besides, activated Akt could further activates downstream effectors S473 and mechanistic target of rapamycin complex 1 and -2 (mTORC1 and mTORC2) leading to growth translation [118, 119] (Fig 27) [120]

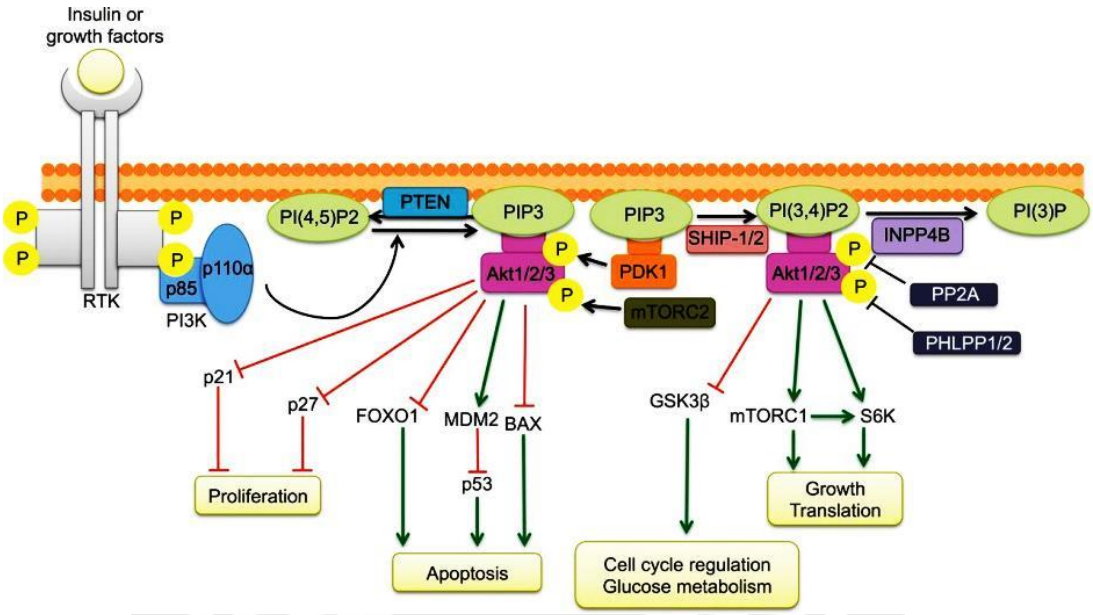

Figure 27 PI3K/AKT signaling pathway

Royal jelly (RJ)

RJ (Fig 28) is a white cream and viscous jelly-like substance that produced and secreted from hypopharyngeal and mandibular gland of the worker bees [121, 122]. It is a special food for a bee destined to become a queen thus also known as a superfood. Currently, RJ is used as commercial medicine product, dietary supplement and cosmetic in many countries. The biological activities of RJ have been reported as anti-tumor, anti-bacterial, anti-allergy, anti-inflammatory, anti-aging, immunomodulatory effect, anti-hypercholesterolemic, vasodilative, nephroprotective and wound healing [123, 124]. RJ consists of water (50-60%), proteins (18%), carbohydrate (15%), lipids (3%-6%), mineral salts (1.5%), and vitamins. Ten-hydroxy-2-decenoic acid (10-HDA) is the major

unsaturated fatty acid component of RJ [125] and 10-HDA content has been applied as a marker for RJ and used for analyzing the quality of RJ [131, 132]

Figure 28 Royal jelly from honey bees

Internet available from: http://en.wikipedia.org/wiki/Royal_jelly

10-Hydroxy -2- Decenoid Acid (10-HDA)

10-HDA is a major unsaturated fatty acid found in royal jelly (RJ). The molecular formula of 10-HDA is $C_{10}H_{18}O_3$ and its molecular weight is 186.3 (Fig 29) [133]. 10-HDA has been reported to be the major bioactive substance in RJ by having various biological activities. The biological effects of 10-HDA have been reported by Blum MS, et al. (1959) and (Takenaka et al. (1986) showing that 10-HDA had a potential of antibacterial by inhibiting growth of *M. pyogenes*, *B..subtilis*, *S. aureus*, *E. coli* and also inhibited growth of fungi (Neurospora sitophila) [134, 135]. In 1994, Fang et al. demonstrated that 10-HDA has anti-inflammatory effect by protecting rats from experimentally induced gastric ulcer [136]. Spannhoff et al. (2011) reported that 10-HDA inhibited histone deacetylase (HDAC) activity throught reactivating the expression of epigenetically

silenced genes in mammalian cells which resulting in the epigenetic regulation of queen-bee developed [137]. Sugiyama et al. (2012) reported that 10-HDA inhibit lipopolysaccharide (LPS)-induced NF-κB activation on in the murine macrophage cell line RAW264 [138]. Zheng et al. (2012) demonstrated the anti-aging activity of 10-HDA by promoting the collagen synthesis and inhibit MMP-1 and MMP-3 expression, leading to prevention of skin photoageing [139-141]. Gasic et al. (2007) and Sugiyama et al. (2013) reported the immunomodulatory activities by 10-HDA that reduced T-cell proliferation and IL-2 production, blocked of LPS-and IFN-β, leads to decrease nitric oxide (NO) production and increased apoptosis [142]. Honda et al. (2015) reported that 10-HDA has the effect on extended the lifespan of the nematode *C. elegans* [143]. Wang et al. (2015) demonstrated that the HDAC was inhibited by 10-HDA on fibroblast-like synoviocytes from rheumatoid arthritis (RA) patients, leading to reducing of the inflammatory response in RA patients [144]. Filipic et al. (2015) showed that 10-HDA inhibited cell proliferation and decreased the level of GSH and significantly increased lipid peroxidation in human colorectal adenocarcinoma cells (CaCo-2) [145]. The report from Makino et al. (2016) indicated that 10-HDA inhibited HDAC activity thought increasing the expression of extracellular superoxide dismutase (EC-SOD) in leukemia THP-1 cells suggesting the possibility of atherosclerosis treatment [146]. Recently report from Peng et al. (2017) has been found that 10-HDA inhibited the synthesis of melanin by acting on tyrosinase enzyme, TRP-1 and TRP-2, and suppressing MITF in the B16F10 melanoma cells, suggesting the development of 10-HDA as a natural melanogenesis inhibitor for the skin cosmetics industry [147] . Interestingly, the report from Izuta et al. (2009) has been reported that 10-HDA could inhibit cell migration, proliferation and VEGF-induced angiogenesis on HUVEC cells. For anticancer effect, 10-HDA inhibits proliferation and decreases glutathione (GSH) level in human colorectal adenocarcinoma cells (CaCo-2). Otherwise, not much has been reported on the anticancer activity of 10-HDA, especially regarding to metastasis inhibition.

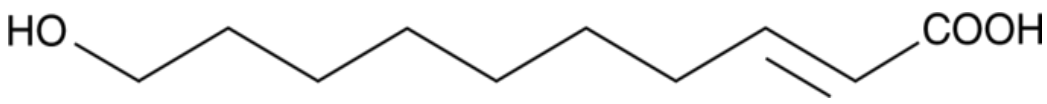

Figure 29 The structure of 10-HDA

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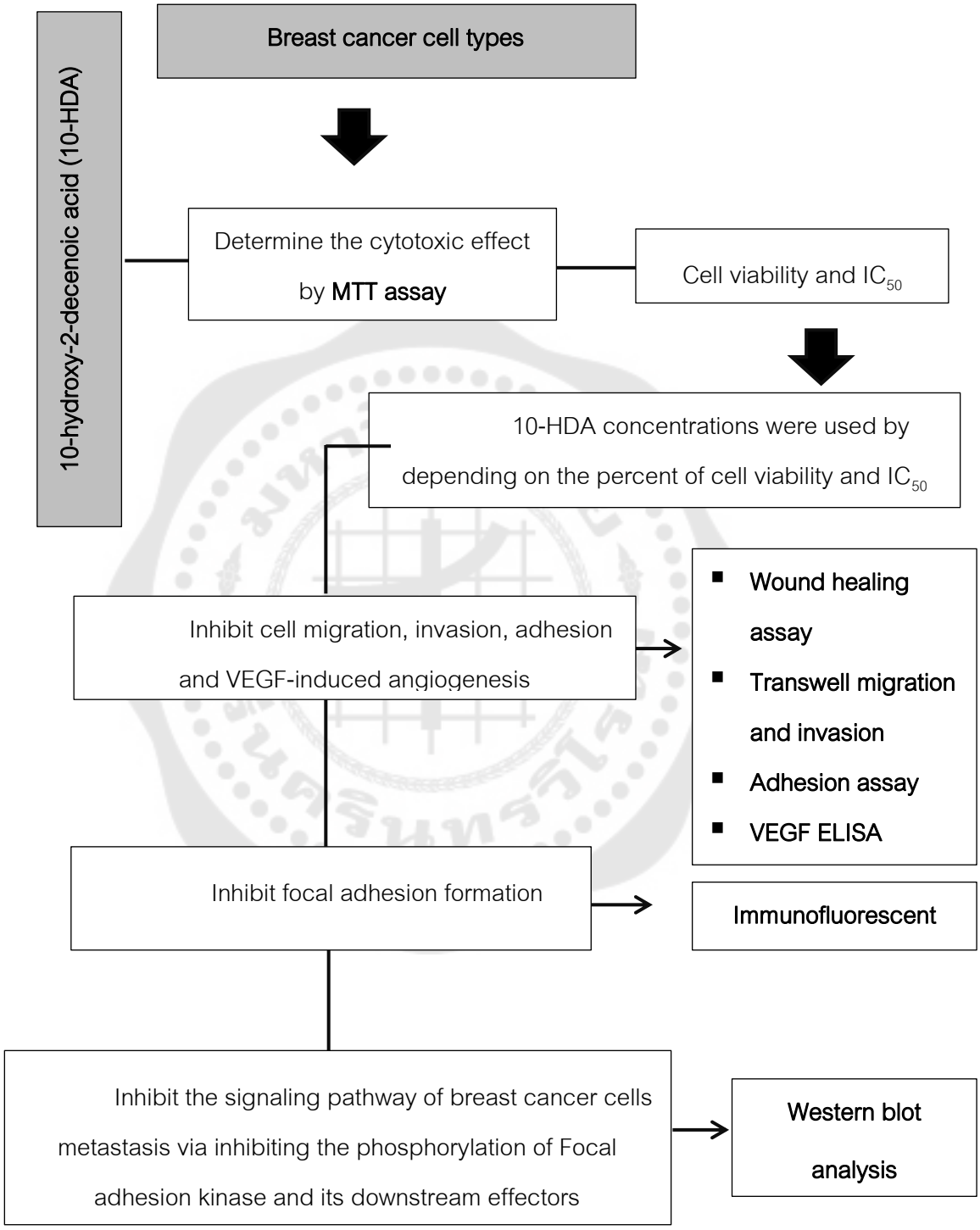
Conceptual framework



CHAPTER III

Materials and methods

Chemicals / Reagents / Kits

Medium 199 (M199), Fetal Bovine Serum (FBS), 0.25% Trypsin-EDTA, Penicillin-streptomycin will be obtained from GIBCO (Invitrogen, USA). Dulbecco's Modified Eagle Medium (DMEM), RPMI1640, DMEM/F12 (1:1 v/v) medium) were obtained from Lonza, USA. Minimum essential medium (MEM), Insulin-transferrin-sodium selenite (ITS), L-glutathione, Dimethyl sulfoxide (DMSO), Hydrogen peroxide solution and fibronectin were obtained from Sigma-Aldrich, Germany. Thiazolyl Blue: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Carl Roth, Germany, 10-HDA was purchased from Cayman Chemical, Michigan USA. Matrigel was purchased from Corning, USA. CHEMICON® ECM Cell Adhesion Array Kit, colorimetric was obtained from Chemicon, Millipore, USA. Human VEGF ELISA Kit was purchased from Abcam, UK. Actin Cytoskeleton and Focal Adhesion Staining (FAK100), Goat anti-Mouse IgG antibody, (H+L) FITC conjugation and protease inhibitor cocktail were purchased from Millipore, USA. ProLong™ Diamond Antifade Mountant and GAPDH were purchased from Invitrogen, USA. Pierce™ ECL Western Blotting Substrate and PageRuler Prestained Protein Ladder were purchased from Thermo Scientific, USA. Lowry Protein Assay was purchased from Bio-Rad, USA. Phospho-FAK (Tyr397), FAK, PI3 Kinase p110α Antibody , Rac1/Cdc42, Phospho-SAPK/JNK (Thr183/Tyr185), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, Phospho-Akt (Ser473), AKT antibody were purchased from Cell signaling, USA. ERK1 and JNK antibody were purchased from Santa Cruz Biotechnology, USA.

Cell cultures

Triple negative breast cancer cells, MDA-MB231, MDA-MB436 and HCC1937 were purchased from LGC standards. African green monkey kidney (Vero) cells were purchased from ATCC. MCF-7, MCF-10A, NIH3T3 (wt) and NIH3T3 expressing constitutively active forms of $BRAF^{V600E}$ were kindly obtained from Prof. Jakob Troppmair, Daniel Swarovski Research Laboratory, Department of Visceral, Transplant, and Thoracic Surgery, Medical University of Innsbruck, Austria.

MDA-MB231, NIH3T3 (wt) and NIH3T3 $BRAF^{V600E}$ were grown in Dulbecco's Modified Eagle Medium (DMEM, Lonza). MCF-7 was maintained in minimum essential medium (Sigma-Aldrich). Vero cells were grown in Medium 199 (Gibco). MDA-MB436 was grown in DMEM and supplemented with 10 µg/mL ITS (insulin-transferrin-sodium selenite, Sigma), and 16 µg/ml L-glutathione (Sigma). MCF-10A was maintained in DMEM/F12 (1:1 v/v) medium (Lonza) and supplemented with 5% fetal bovine serum (FBS), 20 ng/mL EGF, 0.5 µg/µL hydrocortisone, and 10 µg/ml ITS. All media contained 10% FBS, 200 mM L-glutamine, and penicillin (100 U/ml) / streptomycin (100 µg/ml). All cells were maintained at 37 °C in an atmosphere of humidified air with 5% CO2.

Preparation of 10-HDA

10-HDA is a supplied as a crystalline solid with the purity $\geq$ 98% and stored at -20 °C for long term storage. A stock solution of 10-HDA was made by dissolving with DMSO as a solvent.

MTT assay

All cells were suspended and seeded as 100 µl in 96-well plates at a density of 1 x 10^4 cells per well with different condition media of each cell line and incubated overnight. Cells were treated with varying concentrations of 10-HDA at 0.3, 0.6, 1.2, 2.5,

5, 10, 20 mM for 24 hours. Thiazolyl Blue: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Carl Roth, Germany), MTT was mixed with PBS at 5 mg/ml for 100 µL in each well, followed by 100 µl of culture medium as a final concentration of 2.5 mg/ml, and then incubated for 1 h. The formazan crystals formed were dissolved by addition 100 µl of DMSO. The amount of the colored formazan derivative was determined by measuring absorbance at 550 nm with a 620 nm as a reference filter using an Anthos 2010 Microplate Reader. The percentage viability was calculated as: % Viability = [OD of treated cells/OD of control cells] ×100

Wound healing assay

The effect of 10-HDA on the migration of a highly metastasis breast cancer MDA-MB231 cell was determined by monolayer wound healing assay. Cells were grown in 6-well tissue culture plate at a density of 8×10^5 cells per well and starved with serum-free medium overnight. After the cells formed as 90% confluent monolayer, the cells were scratched with 10 µl sterile pipette tip with a similar diameter. Three wounds were made each well and then washed three times with DMEM to remove cell debris. After that, the medium was immediately replaced with or without 10-HDA at 3 and 6 mM. Cell migration was monitored and imaged under inverted microscope at 10x magnification for 0 and 24 h. The distances of wound (compared with control at 0 h) were measured in three-independent wound sites per group. Relative cell motility was calculated as the wound width at 24 h compare to 0 h. The values were calculated at least three independent experiments [148, 149].

Transwell migration and invasion assay

Transwell migration and invasion assay were measured by using Corning transwell inserts (BD Biosciences) with 8.0 µm pore polycarbonate membrane. Transwell

chambers precoated with (invasion) or without (migration) Matrigel [32, 33]. The method was followed by BD Biosciences assay method and Wang P, et al 2014 [138]. Matrigel (BD Biosciences) invasion assay was performed at 37°C dilute with serum-free media at concentration 30 µg/ml. 100 µl of Matrigel was added in 24 well of transwell inserts (8 mm pore size) and incubated 37°C for 2 h. Then, 750 µl of culture media with 10% serum was added to the lower chamber and 200 µl of MDA-MB231 cell suspension containing 2.5 x 10^4 cells/ml in serum-free medium was added in the insert chamber. Then, 10-HDA with varying concentration of 3, and 6 mM was added to the interior of the inserts. The cells were incubated for 24 h at 37°C in 5% CO_2. After that, the medium was removed from the top side of the insert by pipetting out the remaining cell suspension and washed 2 times with PBS. Cells were fixed by 100% ice-cold methanol for 20 min. Then, the methanol was removed and washed 2 times in PBS. Cells were stained with 500 µl of 0.5% crystal violet for 15 min in the lower chamber at room temperature. After that, the insert chamber was removed and washed in distilled water several times or until the dye stop coming off. Non-invasive cells were scraped off by cotton swabs and allowed the insert to air dry. Migrated and invaded cells were photographed by inverted microscope and the stained cells was extracted by 100% methanol and gently rotate on an orbital shaker until the cells-bound stain were completely solubilized. The colorimetric measurement was done by adding 100 µl from extraction stain to 96-well plate with optical density at 540 nm [150].

Adhesion assay

Adhesion assay was performed using CHEMICON® ECM Cell Adhesion Array Kit, colorimetric (Chemicon, Millipore, USA). The plate strips were rehydrated with 200 µl of PBS per well for 10 min at room temperature. After removing the PBS, MDA-MB 231 cells were seeded in each well of the ECM Array plate at a density of 15 x 10^4 cells per

well. Then, 10-HDA was added with concentrations of 0, 0.75, 1.5, 3 and 6 mM and incubated the plate for 2 h at 37°C in 5% CO_2. After incubation, the media was gently discarded or aspirated from the wells and should not allow wells dry. Gently wash each well 2 times with 200 µl per well of Assay Buffer. When removing the wash solution, try to leave about ~25-50 µl per well and the cells were always hydrated. After washing, 100 µl of Cell Stain solution was added to each well and incubated for 5 min at room temperature. Then, the stain solution was removed, gently washed the plate 3 times with deionized water and let the wells air dry for a few minutes. After that, 100 µl of Extraction Buffer was added to each well. Then, the strips were incubated and gently rotated on an orbital shaker at room temperature until the cells-bound stain is completely solubilized, approximately 5-10 min. After that, the plate was measured at OD 540 nm with a microplate reader.

VEGF-A enzyme-linked immunosorbent assay (ELISA)

MDA-MB231 cells were plated in 6-well plate at a density of 1 x 10^6 cells/well in complete medium overnight. After incubated, cells were replaced with 2 ml of serum-free medium containing 3 and 6 mM of 10-HDA for 24 h. The conditioned media were collected and concentrated with Amicon Ultra-4 10K (Millipore) followed by the manufacturer's instruction. Briefly, the device was rinsed with Milli-Q water before use. After that, the sample 4 ml was added to the Amicon® Ultra filter device. Then, place capped filter device into a fixed-angle rotor and spin at 7,500 × g for 20 min. The protein concentrations were measured by the Bradford method. The quantification of VEGF-A was measured according to manufacturer's instructions (VEGFA Human ELISA Kit, Abcam). The samples or each standard were added of 100 µl into VEGF microplate and incubated for 2.5 h at room temperature. The solution was discarded and washed 4 times with 1X Wash Solution. Then, 100 µl of 1X Biotinylated VEGF detection Antibody

was added to each well and incubated for 1 h at room temperature with gentle shaking. After that, the solution was discarded and washed with 1X wash solution. Then, 100 µl of 1X HRP-Streptavidin solution was added to each well and incubate for 45 min at room temperature with gentle shaking. After that, the solution was discarded and washed with 1X Wash Solution. Then, 100 µl of TMB One-Step Substrate Reagent was added to each well and incubate for 30 mins at room temperature in the dark with gentle shaking. 50 µl of Stop Solution was added to each well and read at 450 nm immediately.

Actin cytoskeleton and focal adhesion staining

Actin Cytoskeleton and Focal Adhesion Staining (FAK100, Millipore) was performed following the manufacturer's instruction which the major four steps as follows:

1. Coating slide chamber

Four well slides chambers removable Lab-Tek (Nalge Nunc, Rochester, NY, USA) were used. The slide chambers were coated with 50 µg/ml of fibronectin (Sigma-Aldrich, St. Louis, MO, USA) mixed with 0.2 mg/ml of gelatin (Sigma) for 1 h. Then, the remaining uncoated of fibronectin-gelatin solutions were removed, air dry for 1 h and UV exposure in laminar flow for 30 min.

2. Preparing cells and 10-HDA treatment

Breast cancer MDA-MB231 and MDA-MB436 cells, and normal breast MCF-10A cells were plated at a density of 3 x 10^3 cells/well on the slide chamber and incubated overnight. 10-HDA was treated with 2.5 mM in 24 h and 0.5% DMSO was used as a vehicle control.

3. Fixation and cell permeabilization

Cells were fixed with 4% paraformaldehyde in 1x PBS for 20 min at room temperature. Then, the chambers were washed twice with 1X wash buffer. The permeabilization was done by incubating with 0.1% Triton X-100 in 1x PBS for 5 min at

room temperature and washed twice with 1X wash buffer. Next, blocking solution (1% BSA in 1x PBS) was added for 30 min.

4. Primary and secondary antibody labeling

After removing the blocking solution, cells were incubated with primary antibody (Anti-vinculin) in blocking solution with the dilution of 1:500 and incubated for 1 h. After that, cells were washed three times with 1X wash buffer. Secondary antibody (Goat anti-Mouse IgG antibody, (H+L) FITC conjugation, Millipore) was diluted in 1:200 in 1X PBS and incubated for 1 h at room temperature. For double labeling TRITC conjugated Phalloidin was used in a dilution of 1:100 and incubated simultaneously with the secondary antibody for 1 h at room temperature and washed three times with 1X wash buffer. For nuclei counterstaining, DAPI was used with the dilution of 1:1000 and incubated for 5 min at room temperature and washed three times with 1X wash buffer. Cover slip was mounted by using prolong antifade mounting solution (Invitrogen) and let air dry before visualizing under confocal microscopy with the magnification of the objective lens at 60X.

Western blot analysis

1. Cell seeding and treatment with 10-HDA

MDA-MB231, MDA-MB436, HCC1937, MCF-7, MCF-10a, NIH3T3 (wt) and NIH3T3 $BRAF^{V600E,}$ were plated in 6-well plate which density of 6 x 10^5 cells/well and incubated for 24 h. After that, cells were treated with 1.25, 2.5 and 5 mM of 10-HDA, 0.5% DMSO (negative control), and 0.5 mM H_2O_2 (positive control). The collecting of protein lysate was divided in two groups: 1) treated for 15 min and 2) treated for 24 h.

2. Total protein extraction

Cells were washed twice with cold PBS and added with 150 µL of NP40 lysis buffer containing protease inhibitor (1:100). Then, the cells were incubated on ice on the shaker for 20 min. After that, the cells were scraped with cell scraper and transferred to a fresh eppendorf tube. The lysates were then centrifuged at 16,000 x g

for 20 min at 4°C. Next, the supernatant was transferred to a fresh eppendorf tube and mixed with a 1x Laemmli solution and heated in the heat block at 95 °C for 5 min [4].

3. Protein-determined based on Lowry method

Protein concentration was measured by using Lowry kit from Bio-Rad Dc protein assay kit. Briefly, five µl of the samples without Leammli was mixed with 20 µl of H_2O. Reagent A and S were mixed at a ratio 1:100 and 125 µl were added to the samples and the vortex. One ml of Reagent B was further added to the samples and vortex again. The samples were incubated for 15 to 60 min and measured OD at 750 nm with the cuvettes and NP40 lysis buffer was used as blank control [151].

4. SDS-PAGE and immunoblotting

To separate the protein samples, SDS-PAGE was used. Up to 30 µg of samples were loaded at 10% and 12.5% gels depend on the size of proteins. PageRuler Prestained Protein Ladder (Thermo Scientific) was used as the protein marker. The gels were run with the running buffer at 50 volts for 5 min as the first step and 150 volts for 55 min as a second step. After that, the proteins were wet transferred to nitrocellulose membrane by putting 1) black panel, 2) filter pad, 3) 2 pieces of Whatman 3 MM filter paper, 4) SDS gel, 5) nitrocellulose membrane, 6) 2 pieces of Whatman 3 MM filter paper, 7) filter pad and 8) white panel. The blotting was done at 0.4 ampere for 60 min. The membranes were washed with 1X TBST for 5 min and blocked with 5% TBSTM for 60 min. After that, the membranes were incubated overnight at 4°C with primary antibody. After that, membranes were washed for 5 min (3X) in TBST and incubated with secondary antibody for 60 min on a shaker at room temperature. Then, membranes were washed further for 5 min (5X) in TBST, and visualized by ECL chemiluminescent (Thermo Scientific). Films were scanned and analyzed band intensity by using the ImageJ (NIH) software.

Statistical analysis

The results were expressed as mean ± standard error (SD, for each group n=3). All data were processed with GraphPad Prism 5 software. Data analysis was

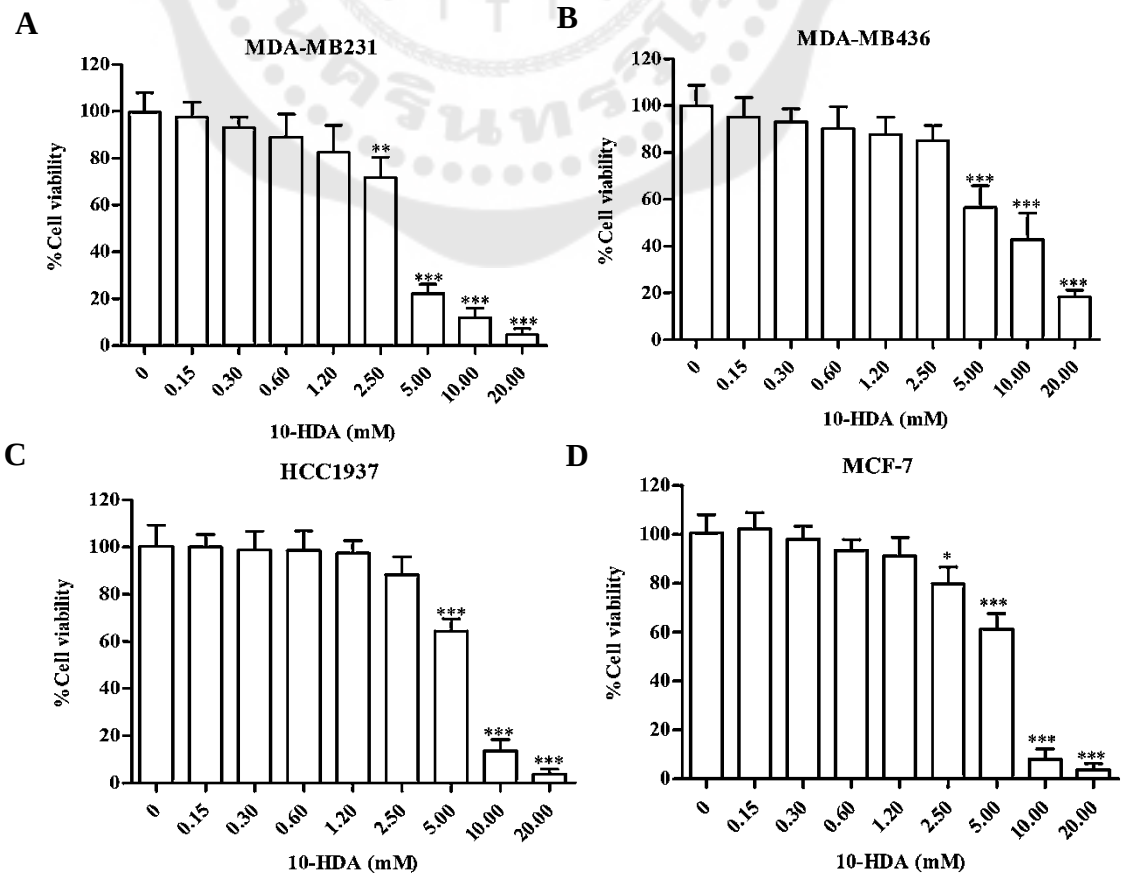
performed by using nonlinear regression to evaluate the IC50. Statistical significance was assessed by One-way Analysis of Variance (ANOVA), analyzed for adhesion, migration and invasion assay. Two-way ANOVA was used for wound healing and evaluated for the significance of differences between the groups, which considered at $p \leq 0.05$. Specific p-values are indicated in figure legends.

CHAPTER IV

RESULTS

1. The cytotoxic effect of 10-HDA on breast cancer cells line

The effect of 10-HDA, purified from RJ, on the viability of triple negative receptors of human breast cancer cell line, MDA-MB231, MDA-MB436 and HCC1937, and ER-positive human breast cancer cells MCF-7 were examined by MTT assay. MCF-10A, Vero, NIH 3T3 (wt) and NIH3T3 $BRAF^{V600E}$ cell line were used as a control cell group. All cells were incubated for 24 h with concentrations ranging from 0 mM to 20 mM of 10-HDA and used 1% DMSO as a vehicle control. As shown in Fig. 30, 10-HDA treated on MDA-MB231, MDA-MB436, HCC1937 and MCF-7 have shown the IC_{50} valued at 3.5, 5.1, 5.26 and 5.2 mM respectively. While for MCF-10A, Vero cells, NIH3T3 and the NIH3T3 $BRAF^{V600E}$ cell line were found at 5, 18.5, 10 and 7 mM. The vehicle control had no effect on cell survival.



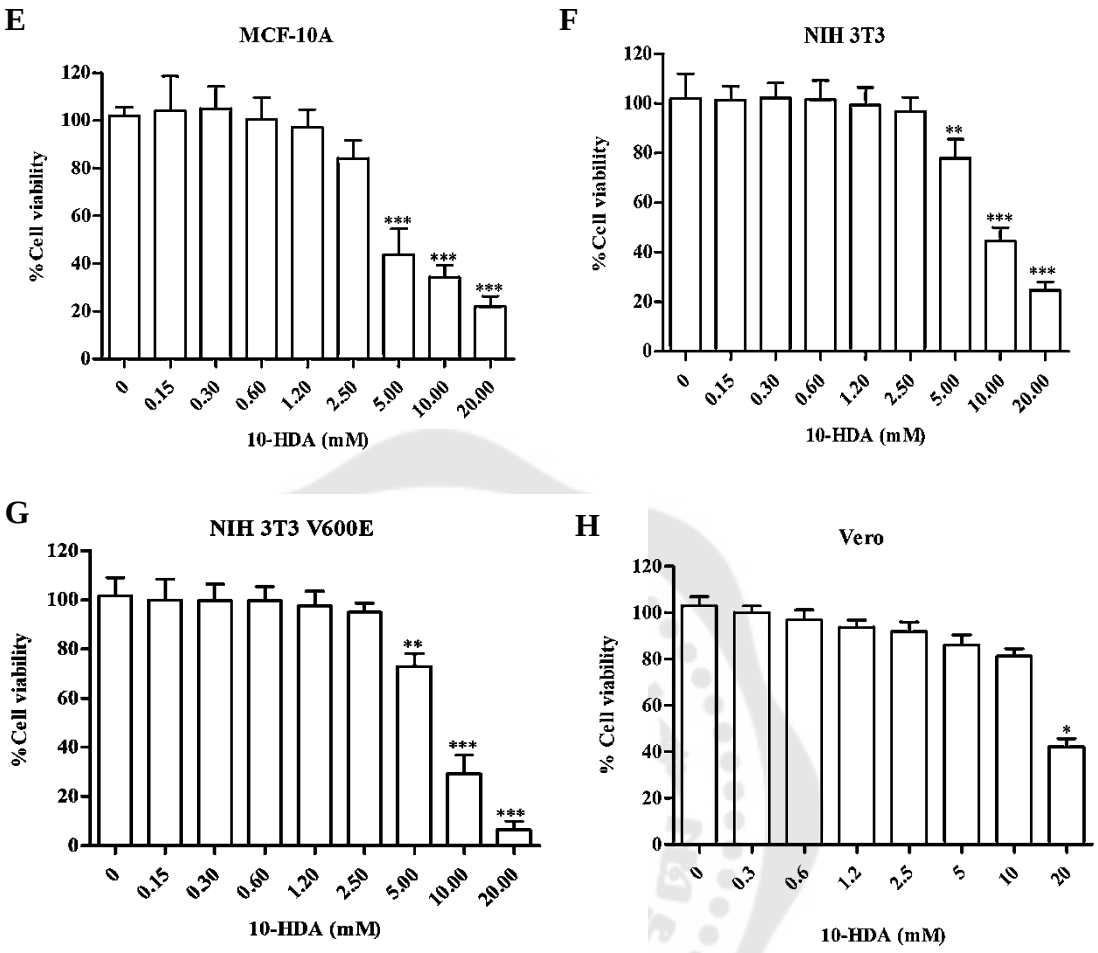


Figure 30 The effect of 10-HDA on cell viability was assessed by MTT assay

The effect of 10-HDA on cell viability was assessed by MTT assay. The cancer cells used were triple negative of human breast cancer cell lines MDA-MB231 (A), MDA-MB436 (B) and HCC1937(C), ER positive of human breast cancer MCF-7 (D). While a control cells were human normal breast MCF-10A (E), Fibroblast cell NIH3T3 (F), NIH3T3 BRAFV600E (G) and Vero cell line (H). All samples were treated with 10-HDA at 0, 0.3, 0.6, 1.2, 2.5, 5.0, 10.0 and 20.0 mM for 24 h. Each bar represents the mean ± SD of three independent experiments and the data are expressed as percentage of cell viability. ***$p<0.001$, **$p<0.01$ and *$p<0.05$ are significantly different compared with vehicle control.

2. 10-HDA reduces breast cancer cell migration and invasion

To evaluate the effect of 10-HDA on cell migration and invasion, a wound healing assay was first performed. The monolayer of MDA-MB231 cells was scratched and treated with 3 mM and 6 mM of 10-HDA and the effect measured after 0 and 24 h. As shown in Fig. 31A, the blockage of cell migration was clearly observed in the 10-HDA treated samples. The wound width with regard to time and 10-HDA concentration is shown in Fig. 31B which indicates that 10-HDA significantly inhibited cell migration in a dose-dependent manner. To analyze the chemotactic response of cell migration, a transwell migration assay was performed by treating MDA-MB231 with 10-HDA at concentrations of 0, 3, 6 mM for 24 h. As depicted in Fig. 32A-B, the number of MDA-MB231 that moved through the transwell insert to the lower chamber was significantly reduced dose-dependently. For invasion, the transwell was pre-coated with Matrigel as a source of extracellular matrix (ECM) proteins on the upper insert before seeding with cancer cells and treated with 10-HDA. The FBS was added in the lower chamber as the chemoattractant to strongly induce cancer cells to invade the lower part. As demonstrated in Fig. 33A-B, 10-HDA significantly reduced cancer cell invasion in a dose-dependent manner compared to the untreated control cells. These results indicate that 10-HDA clearly has an inhibitory effect on breast cancer cell migration and invasion.

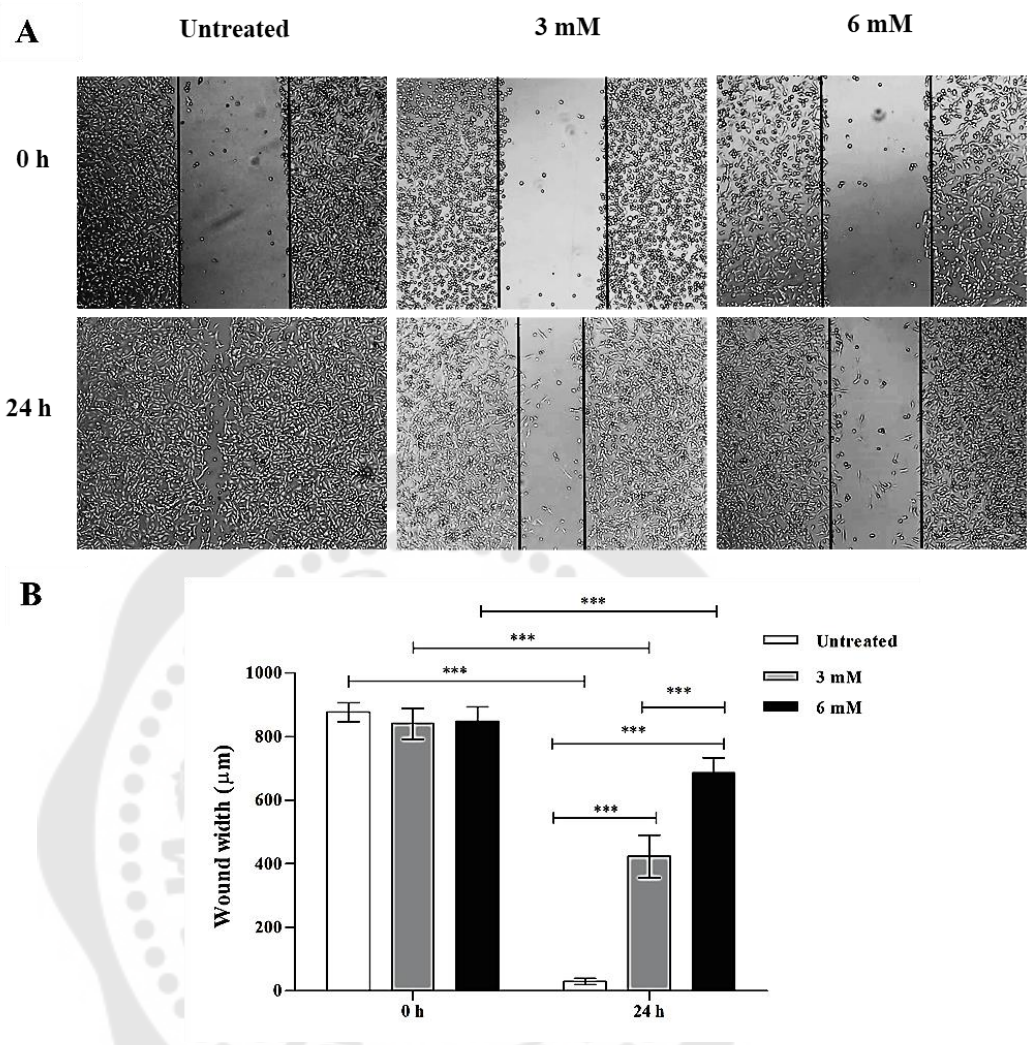


Figure 31 The effect of 10-HDA on breast cancer cell migration

Evaluated by wound healing assay. Confluent MDA-MB-231 cells were scratched and treated with 0, 3 or 6 mM 10-HDA for 24 h. The morphology (A) and the wound width (B) of untreated and treated samples were measured at least three locations in each wound, and the final data are shown as the mean ± SD of three independent experiments. Two-way ANOVA was used for evaluating the significance of differences between the groups and within each group. ***Significantly different from the control ($p < 0.001$).

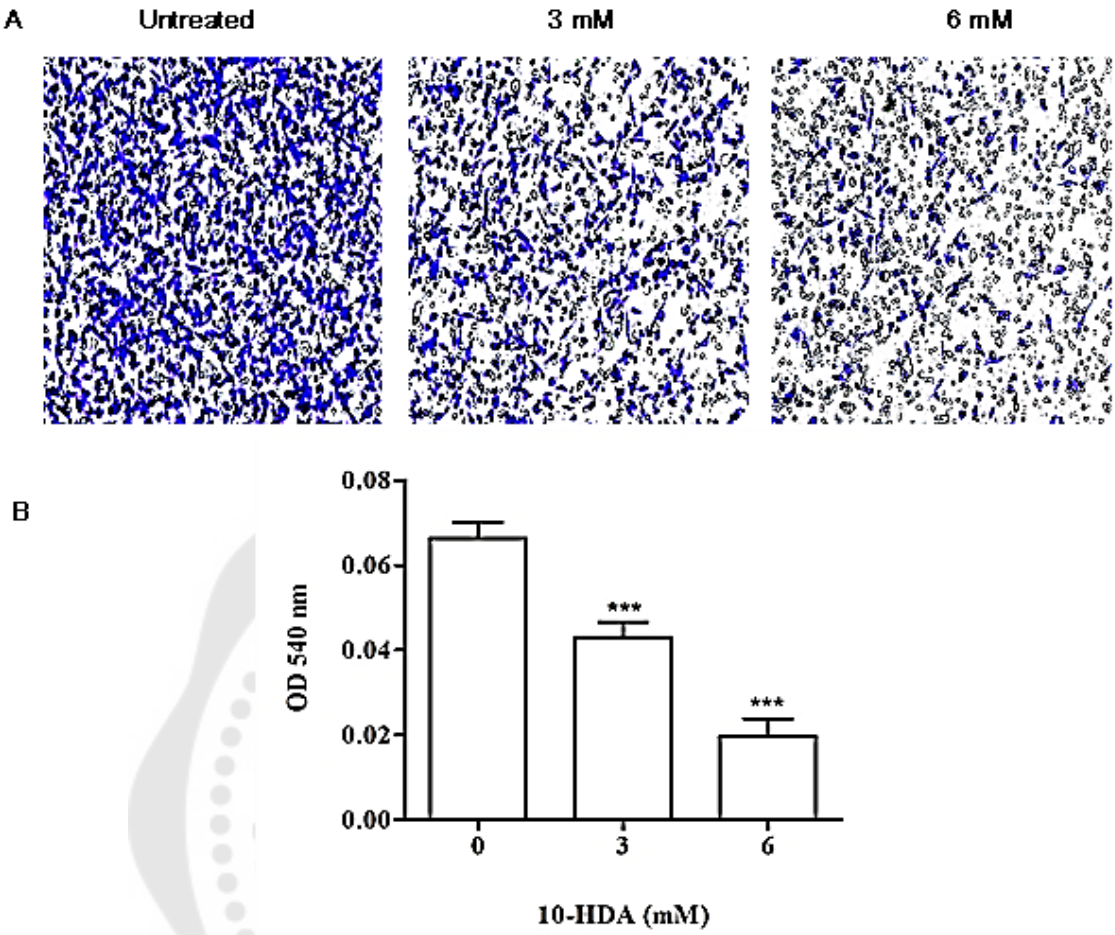


Figure 32 10-HDA affected the ability of breast cancer cell migration

Transwell assay was performed by seeding MDA-MB231 cells on the upper inserts and treated with 10-HDA at 0, 3 and 6 mM for 24 h. While the lower chambers were loaded with 10% of FBS as a chemoattractant. The morphology of cancer cells migrated from the upper inserts pass through the lower chambers was fixed with methanol, stained with crystal violet and photographed (A). The migrated cells were quantified by using methanol extraction and measured OD at 540 nm (B). The data are expressed as mean ± SD, n=3. ***$p < 0.001$ compared with vehicle control samples.

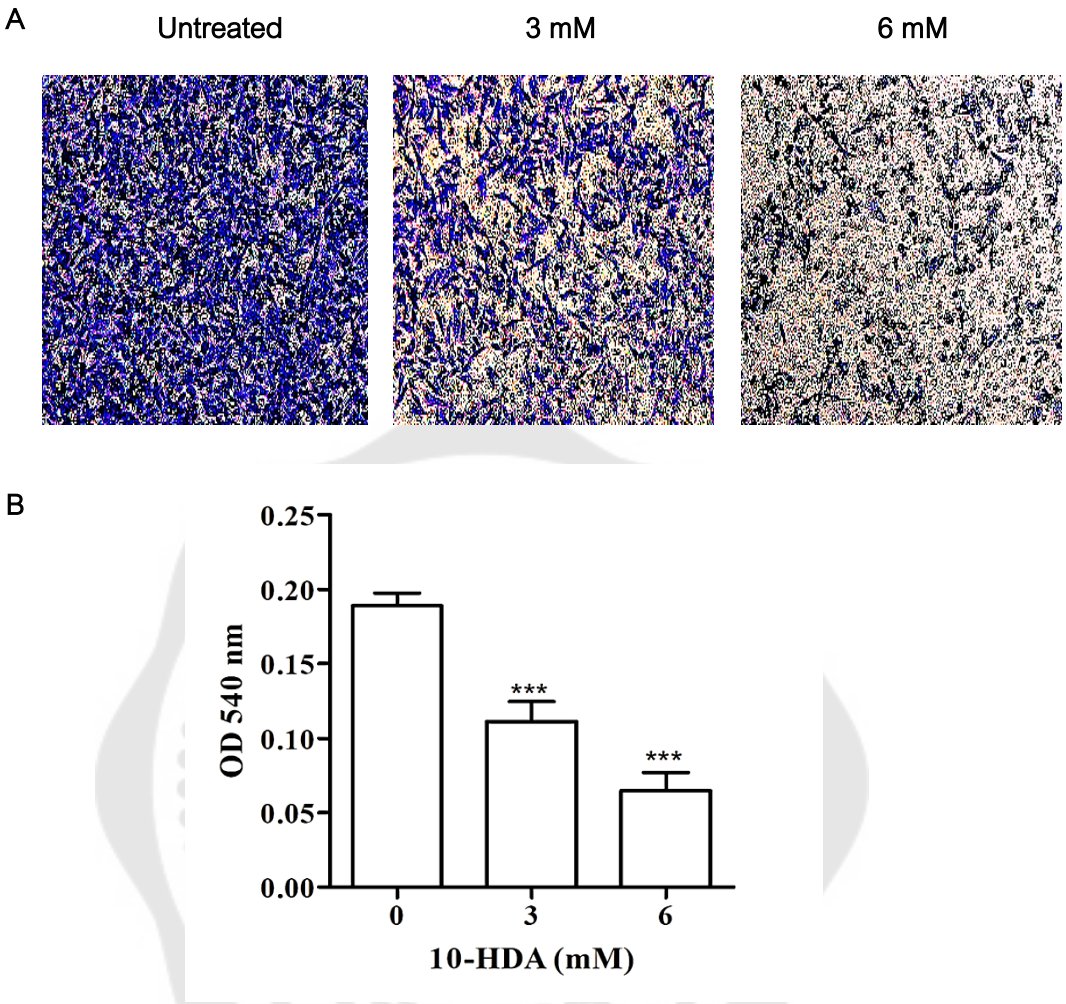


Figure 33 Breast cancer cell invasion was inhibited by 10-HDA

The experiment was done as illustrated for transwell assay, except the upper inserts were pre-coated with Matrigel prior to perform the experiment. The morphology of cancer cells invaded from the upper inserts through the Matrigel to the lower chambers were fixed with methanol, stained with crystal violet and photographed (A). The invaded cells were quantified by using methanol extraction and measured OD at 540 nm (B). The data are expressed as mean ± SD, n=3. ***p< 0.001, compared with vehicle control samples.

3. Anti-adhesion effect of 10-HDA

Cell adhesion is one important step of cancer cell metastasis. In order to determine the effect of 10-HDA on adhesion of highly metastatic breast cancer MDA-MB231, the in vitro ECM cell adhesion array assay was performed. Different ECM proteins; collagen I, collagen II, collagen IV, fibronectin, laminin, tenascin and vitronectin were pre-coated before treatment with 10-HDA at 0.75, 1.5, 3 and 6 mM for 2 h. The effect of 10-HDA as anti-adhesion agent was significantly demonstrated as the number of cell adhesions declined at the concentrations of 3 and 6 mM, and 1.5 mM compared to untreated cells (Fig. 34). Interestingly, the inhibitory effect of 10-HDA was clearly seen from 0.75 mM for the binding of collagen IV, fibronectin and vitronectin compared to other ECM proteins. These data suggested that 10-HDA has a strong effect on the adhesion of a highly metastasis-MDA-MB231breast cancer cells by acting on specific ECM component proteins.

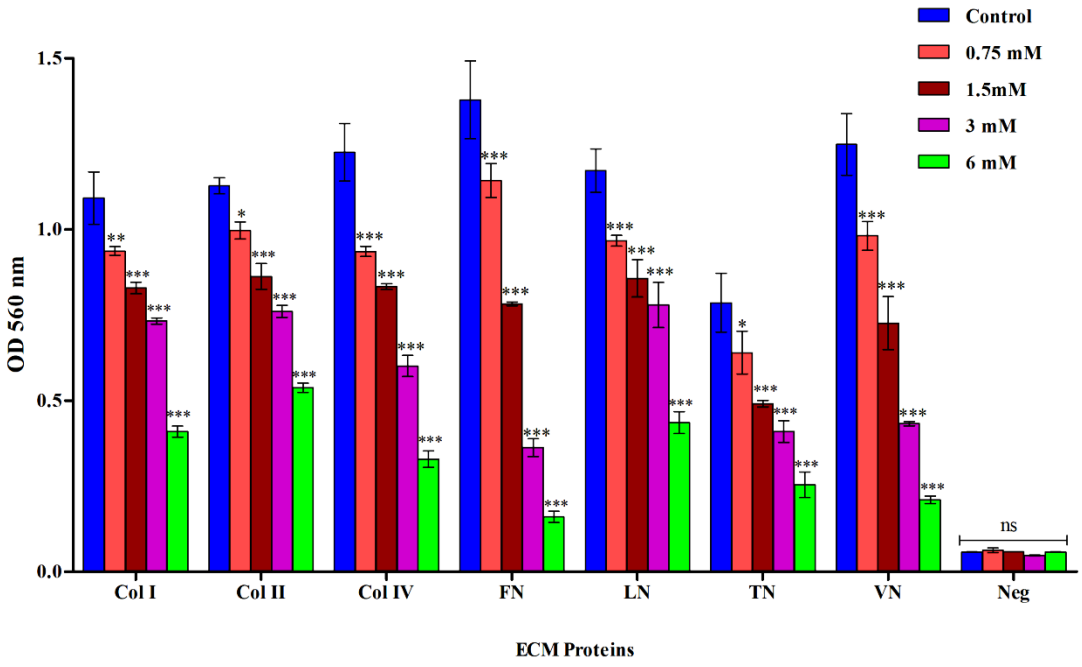


Figure 34 Inhibition of breast cancer cell adhesion by 10-HDA

ECM array plates coated with collagen I (Col I), collagen II (Col II), collagen IV (Col IV), fibronectin (FN), laminin (LN), tenascin (TN), or vitronectin (VN) were seeded with MDA-MB-231 cells and treated with 10-HDA at 0, 0.75, 1.5, 3 and 6 mM for 2 h. Adherent cells were stained and quantified by extracting the stain and measuring the OD at 560 nm. The data are represented as the mean ± SD, n=3. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to the vehicle control.

4. Effect of 10-HDA on VEGF-A induced angiogenesis of MDA-MB231 cells

The production of VEGF is a crucial step of physiological and pathological angiogenesis. VEGF induces the formation of new blood vessels, maintenance of blood vessel structure and also helps in wound healing. Amongst VEGF, VEGF-A plays the major role in tumor angiogenesis and helps in cancer progression and survival. So, in order to observe the effect of 10-HDA on the angiogenic process, the release of VEGF-A from the cancer cells was evaluated. The culture medium of treated samples was collected, concentrated and identified by VEGF-A Human ELISA assay. As shown in Fig. 35, the secretion of VEGF-A was significantly decreased in 10-HDA treated samples in dose-dependent manner (***$p<0.001$ and **$p<0.01$) compared to the untreated sample. This result may suggest that 10-HDA has an anti-angiogenic effect on highly metastatic breast cancer cells.

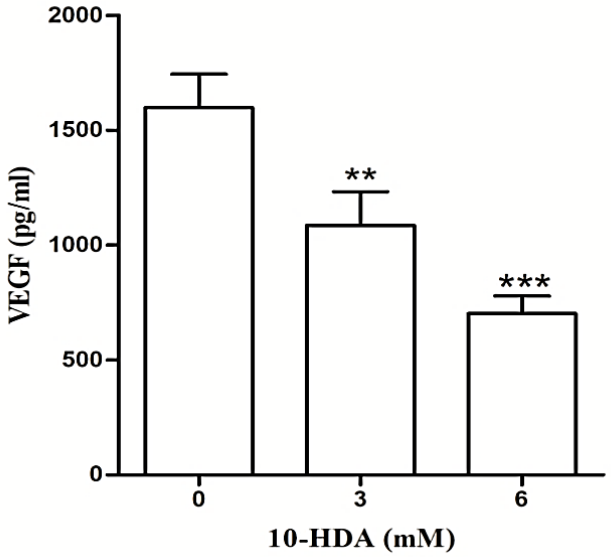


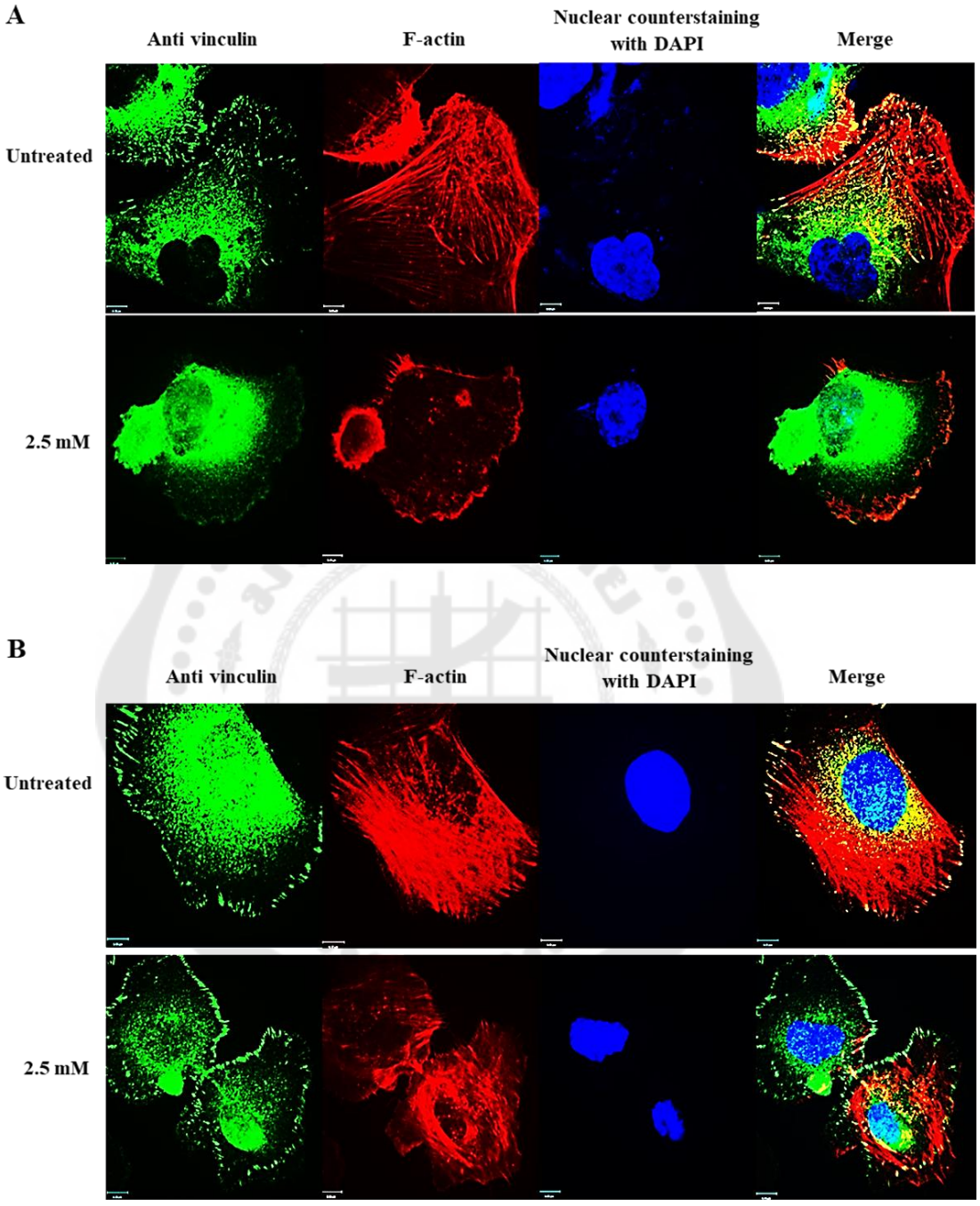
Figure 35 VEGF release from breast cancer cells is inhibited by 10-HDA

MDA-MB231 cells were treated with varying concentrations of 10-HDA for 24 h. Culture supernatants were collected, concentrated and the protein concentration measured. The data are expressed as mean ± SD, n=3. $^{**}p < 0.01$ and $^{*}p<0.05$ compared to vehicle control.

5. 10-HDA inhibit co-localization of vinculin and actin cytoskeleton at focal adhesion complex

Co-localization of vinculin and actin cytoskeleton proteins is known as a marker of focal adhesion complex formation for cancer cell migration. To evaluate the effect of 10-HDA on the formation of focal adhesion complex, two types of TNBC with the highly metastatic property, MDA-MB231 and MDA-MB436 were selected and compared to the untransformed breast epithelial cell line- MCF10A. Treatment was carried out with 2.5 mM of 10-HDA for 24 h. The localization was determined by confocal microscopy, in which the green signal is from vinculin and the red signal from actin cytoskeleton. DAPI was used for nuclear staining. The co-localization was shown as a yellow dot in the merged image. As depicted in Fig 36, the distribution of vinculin in the untreated cells was observed around the nucleus and scattered at the membrane periphery, while actin

demonstrated as stretched-filaments along the cytoplasmic area and attached to the plasma membrane. These characteristics were similar both in the TNBCs MDA-MB231 (36A) and MDA-MB436 (36B) and the untransformed breast cells MCF-10A (36C). The co-localization of vinculin and actin (yellow dots) in both TNBCs were observed at the membrane periphery in which the amount seems to be decreased in treated samples of all three cell types. In addition, it was noticed that the cell structure of 10-HDA treated samples was more rounded than the untreated cells, both the TNBCs and the untransformed cells indicating that the structure of migrating cells was inhibited. This data supports that 10-HDA has the ability to inhibit the formation of focal adhesion complexes which is the marker for cancer cell migration.


A
Anti vinculin
F-actin
Nuclear counterstaining with DAPI
Merge
Untreated
2.5 mM
B
Anti vinculin
F-actin
Nuclear counterstaining with DAPI
Merge
Untreated
2.5 mM

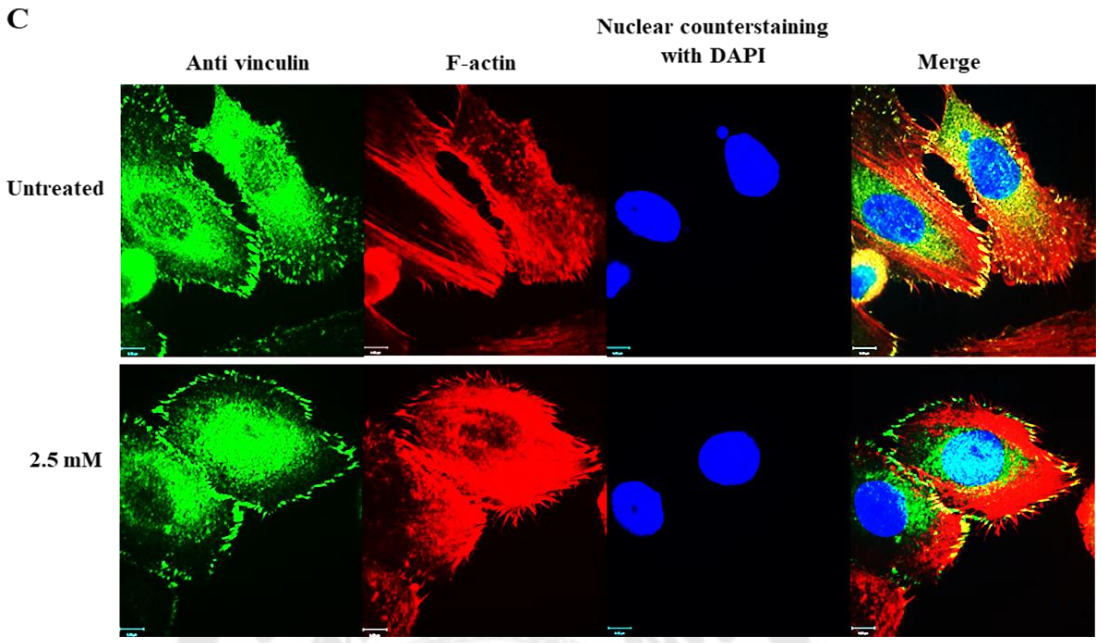


Figure 36 10-HDA inhibited the co-localization of focal adhesion proteins

Immunocytochemistry was performed to investigate the focal adhesions: Vinculin (green), F-actin (red) and nucleus (blue) are shown. The co-localization is depicted yellow dots. 10-HDA treatment was carried out with 2.5 mM for 24 h on MDA-MB231 (A), MDA-MB436 (B) and MCF10A cells (C). The data are representative of three-dependent experiments.

6. The signaling pathway of FAK and its downstream effectors

FAK has been implicated in many cancer types [(1)]. Overexpression of FAK has been indicated in aggressive and highly metastatic cancers [(2)]. To determine the effect of 10-HDA on the role of FAK as an upstream effector of signal transduction in cancer metastasis, the phosphorylation of FAK was investigated. Three types of TNBC (MDA-MB231, MDA-MB436 and HCC1937) and ER+ breast cancer (MCF-7) as well as normal epithelial breast cell line (MCF10A), fibroblast cell line NIH3T3 (wt) and NIH3T3 $BRAF^{V600E}$ (mutant) were studied. Cells were treated with different concentrations of 10-

HDA and the results compared with the untreated cell, vehicle control (as a negative control) and H_2O_2 as a positive cytotoxic inducer. Also, the time-course of signaling was determined by treatment for a short (15 min) and a long period (24 h). The specific antibodies were used for analysis of FAK, PI3-K, RAC1, Akt, ERK, JNK and P38, while GAPDH was used as loading control equal amount of protein. The results of each cell lines; MDA-MB231, MDA-MB436, HCC1937, MCF-7, MCF-10A, NIH3T3 and NIH3T3 $BRAF^{V600E}$ were depicted in Fig. 36-42, respectively. The results of FAK, PI3-K, RAC1, Akt, ERK1/2, JNK and P38 were concluded as shown in the table 2, while the data can be explained as followings.

6.1 10-HDA affects cell migration and invasion through FAK/PI3-K/RAC1 pathway

10-HDA treated cells were significantly inhibited the phosphorylation of FAK and subsequently inhibited PI3-K and RAC1 activation in all three types of TNBC (MDA-MB231, MDA-MB436 and HCC1937; Fig 37-39) at 24 h compared to the early response. The inhibition was clearly seen from the concentration at 2.5 mM of 10-HDA. This effect was less observed in MCF-7 cells (Fig.40) and NIH3T3 $BRAF^{V600E}$ (Fig.41), while not significantly noticed in MCF10A cells (Fig.42) and NIH3T3 cells (Fig.43). Therefore, the results indicated that 10-HDA specifically inhibits the phosphorylation of FAK in TNBCs and suggested that 10-HDA affected cancer cell motility via the signaling of the FAK/PI3-K/RAC1 pathway.

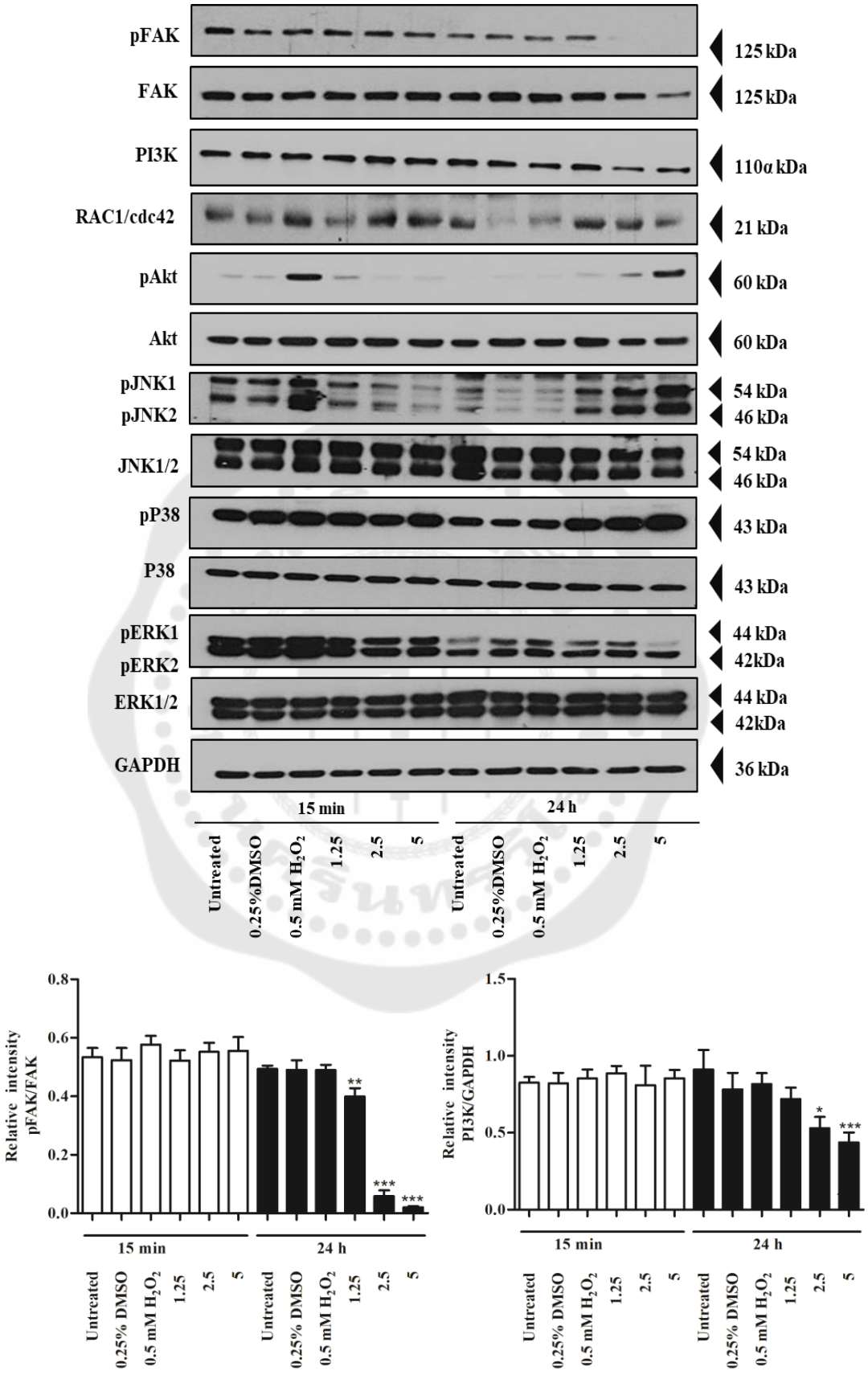
6.2 10-HDA inhibits cell proliferation through FAK/ERK signaling

FAK has been shown to involve with several signal transduction such as cell motility, survival, adhesion and proliferation. The signaling of FAK on the process of cell proliferation has been shown to induce mainly in two ways as FAK/PI3-K/Akt [152-154] or FAK/ERK pathway [155, 156]. As demonstrated in Fig 37-43, 10-HDA treated samples decreased FAK phosphorylation and subsequently inhibited PI3-K activation. However, the phosphorylation of Akt was significantly reduced only in MCF-10A, NIH3T3 and

NIH3T3 $BRAF^{V600E}$. In all TNBCs samples, the level of Akt phosphorylation was in the opposite direction of the results of PI3-K. This data indicated that the function of Akt in TNBC were not under PI3-K signaling. The reduction of PI3-K was more related to ERK, as the level of ERK phosphorylation significantly reduced in all TNBCs. Therefore, these results suggested that 10-HDA has ability to inhibit TNBCs' proliferation through the signal transduction of FAK/ERK pathway and not rely on FAK/PI3-K/Akt pathway.

6.3 10-HDA affects the phosphorylation of Akt related to MAPKs activation and induces apoptosis

As it is known that Akt is the downstream effector of PI3-K that regulates cell survival and anti-apoptosis in many cancers, so the level of PI3-K and Akt should reduce under the anti-proliferation effect of 10-HDA. However, the results found that the level of Akt significantly increased in expression, while PI3-K was reduced and this effect were observed only in breast cancer types and mutant forms of $BRAF^{V600E}$ in NIH3T3 cells. To further investigate the correlation of Akt results with other downstream mediators, it was shown that the level of JNK and P38/MAPK were increased in the same direction as Akt in TNBCs and NIH3T3 $BRAF^{V600E}$. Moreover, 10-HDA was shown no significant difference (nsd) to induce the phosphorylation of JNK and P38 in MCF-7, MCF-10A and NIH3T3. Therefore, this result implies that 10-HDA may induce apoptosis of TNBCs via activating Akt, and subsequently involving with JNK and P38 pathway.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PI3K
RAC1/cdc42
pAkt
Akt
pJNK1
pJNK2
JNK1/2
pP38
P38
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pERK2
ERK1/2
GAPDH
125 kDa
125 kDa
110α kDa
21 kDa
60 kDa
60 kDa
54 kDa
46 kDa
54 kDa
46 kDa
43 kDa
43 kDa
44 kDa
42kDa
44 kDa
42kDa
36 kDa
15 min
24 h
Untreated
0.25%DMSO
0.5 mM H2O2
1.25
2.5
5
Relative intensity pFAK/FAK
Relative intensity PI3K/GAPDH
0.0
0.2
0.4
0.6
0.8
0.5
1.0
1.5
**
***
*

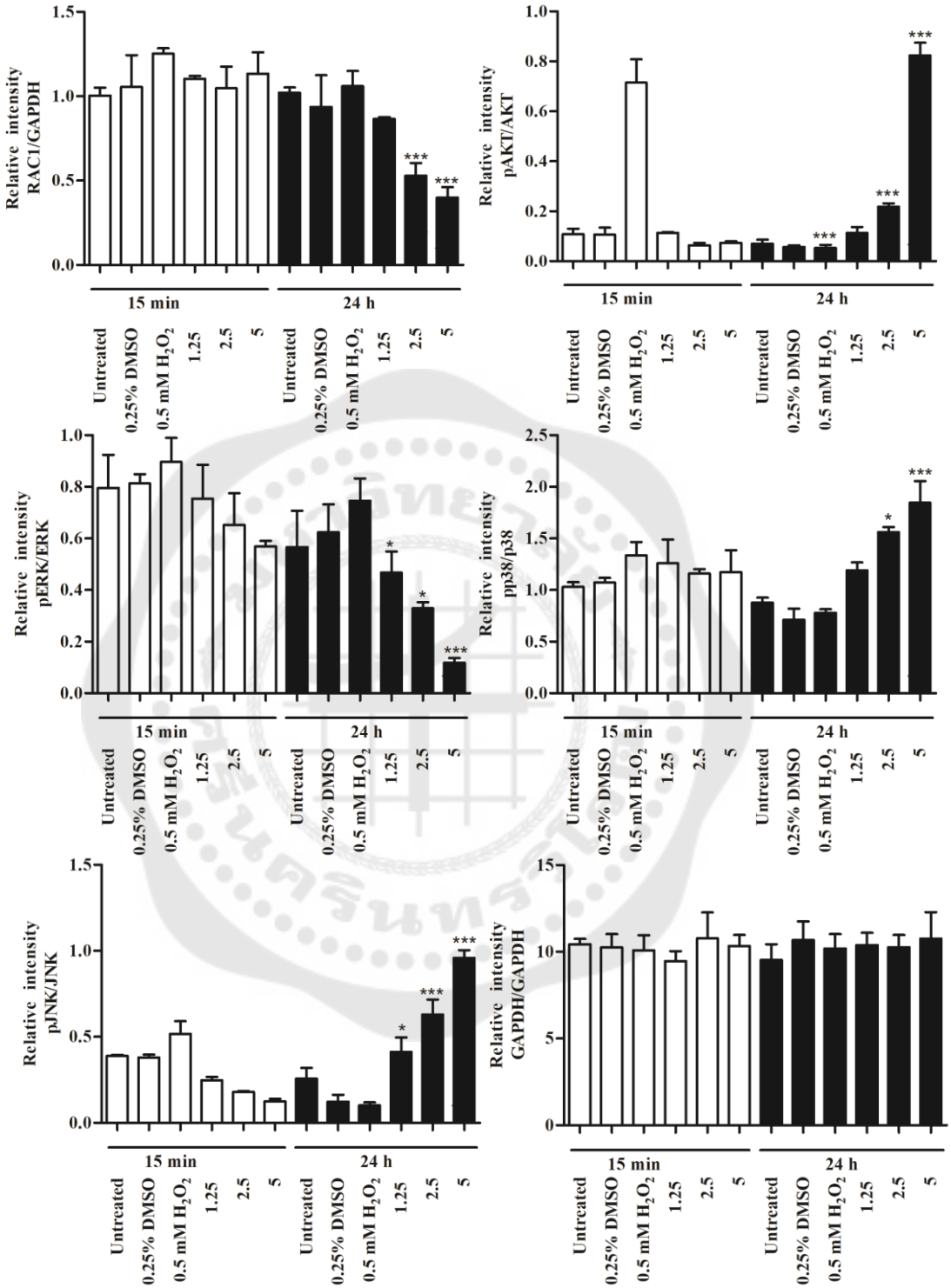
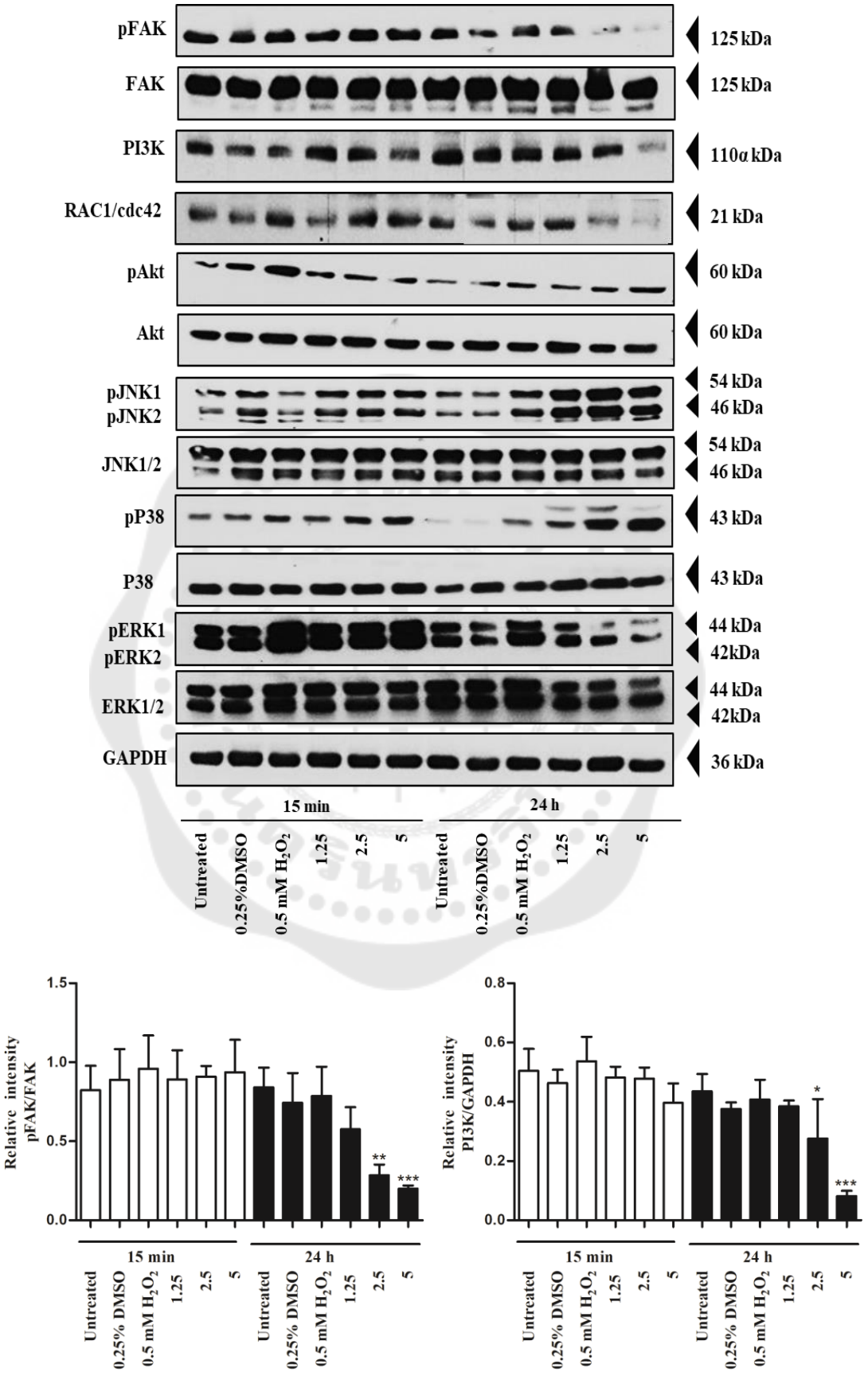


Figure 37 Western blot analysis of MDA-MB231 treated with 10-HDA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FAK
125 kDa
PI3K
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RAC1/cdc42
21 kDa
pAkt
60 kDa
Akt
60 kDa
pJNK1
pJNK2
54 kDa
46 kDa
JNK1/2
54 kDa
46 kDa
pP38
43 kDa
P38
43 kDa
pERK1
pERK2
44 kDa
42kDa
ERK1/2
44 kDa
42kDa
GAPDH
36 kDa
15 min
24 h
Untreated
0.25%DMSO
0.5 mM H2O2
1.25
2.5
5
Relative intensity pFAK/FAK
Relative intensity PI3K/GAPDH
**
***
*

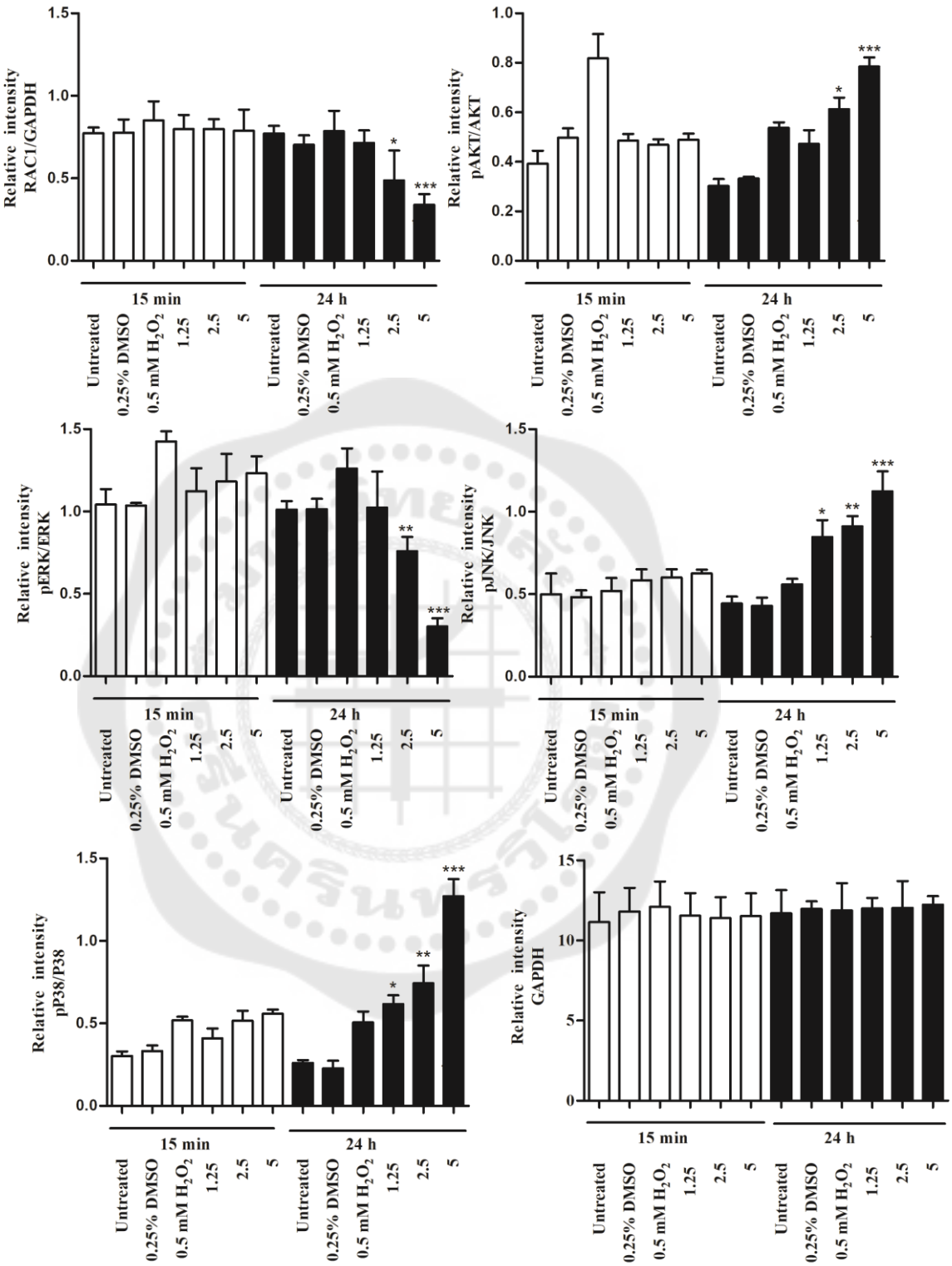
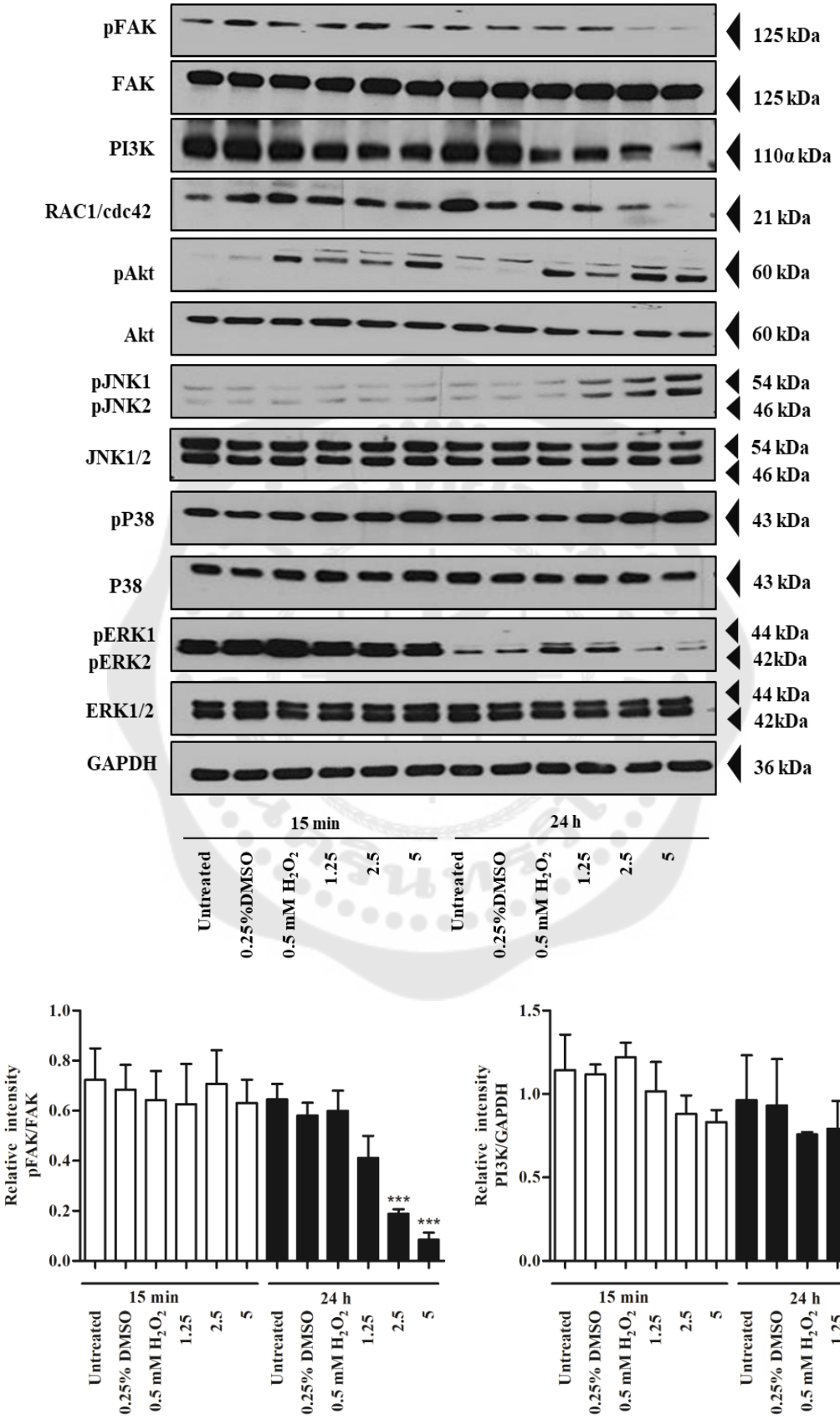


Figure 38 Western blot analysis of MDA-MB436 treated with 10-HDA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Akt
pJNK1
pJNK2
JNK1/2
pP38
P38
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pERK2
ERK1/2
GAPDH
125 kDa
125 kDa
110α kDa
21 kDa
60 kDa
60 kDa
54 kDa
46 kDa
54 kDa
46 kDa
43 kDa
43 kDa
44 kDa
42kDa
44 kDa
42kDa
36 kDa
15 min
24 h
Untreated
0.25%DMSO
0.5 mM H2O2
1.25
2.5
5
Relative intensity pFAK/FAK
Relative intensity PI3K/GAPDH
***
*
**

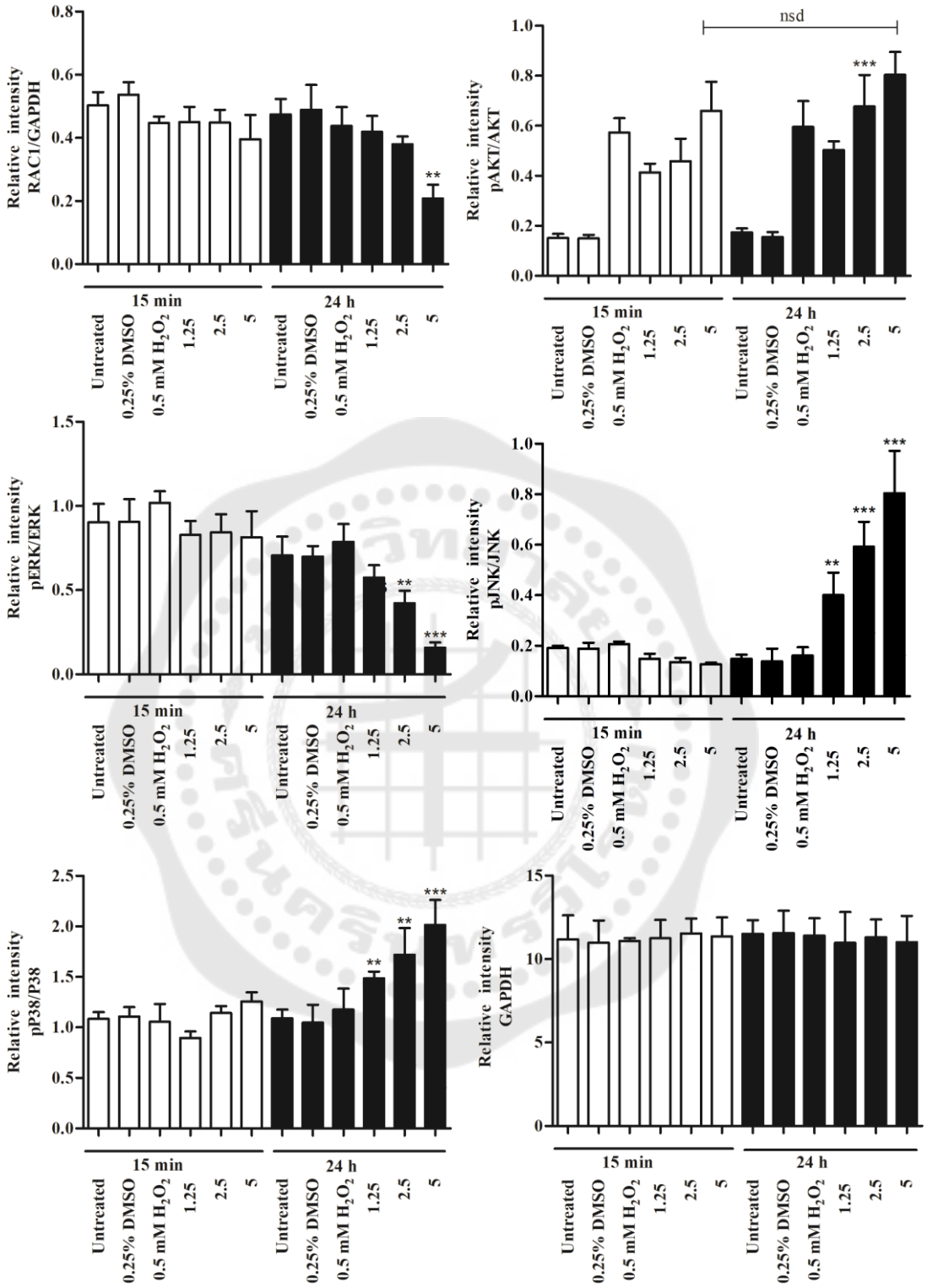
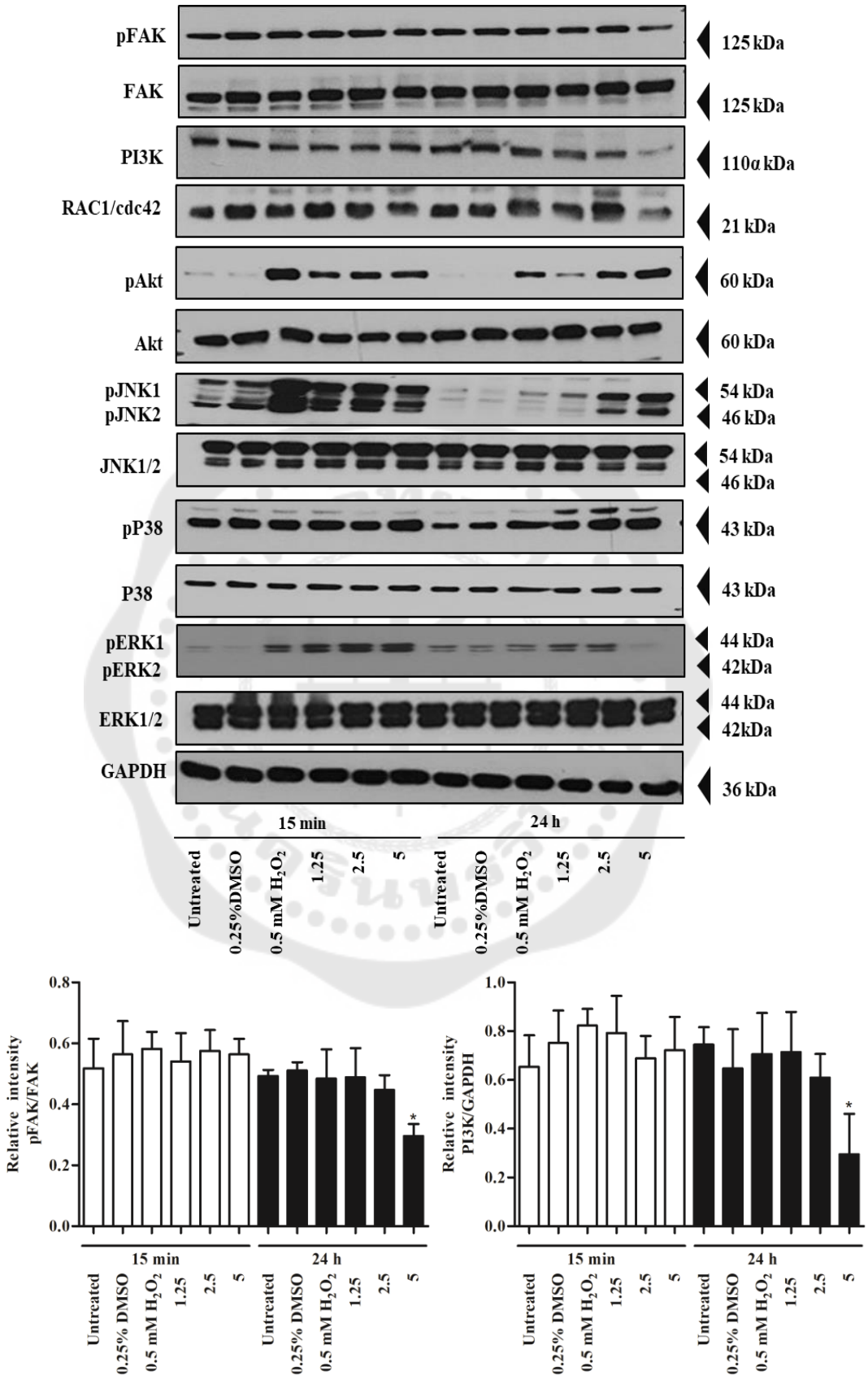


Figure 39 Western blot analysis of HCC1937 treated with 10-HDA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Akt
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pJNK2
JNK1/2
pP38
P38
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pERK2
ERK1/2
GAPDH
125 kDa
125 kDa
110α kDa
21 kDa
60 kDa
60 kDa
54 kDa
46 kDa
54 kDa
46 kDa
43 kDa
43 kDa
44 kDa
42kDa
44 kDa
42kDa
36 kDa
15 min
24 h
Untreated
0.25%DMSO
0.5 mM H2O2
1.25
2.5
5
Relative intensity pFAK/FAK
Relative intensity PI3K/GAPDH
0.0
0.2
0.4
0.6
0.8
1.0
*

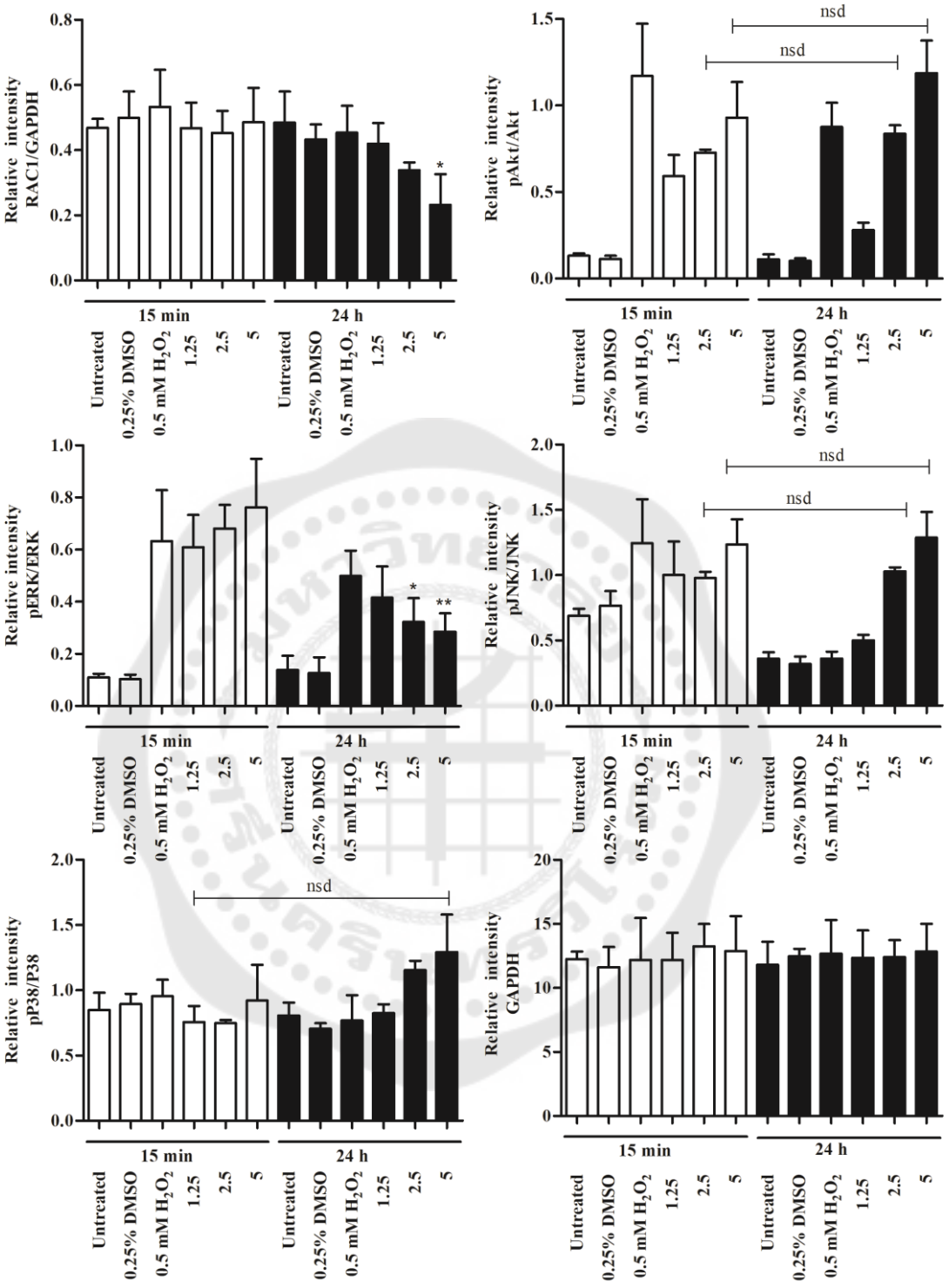
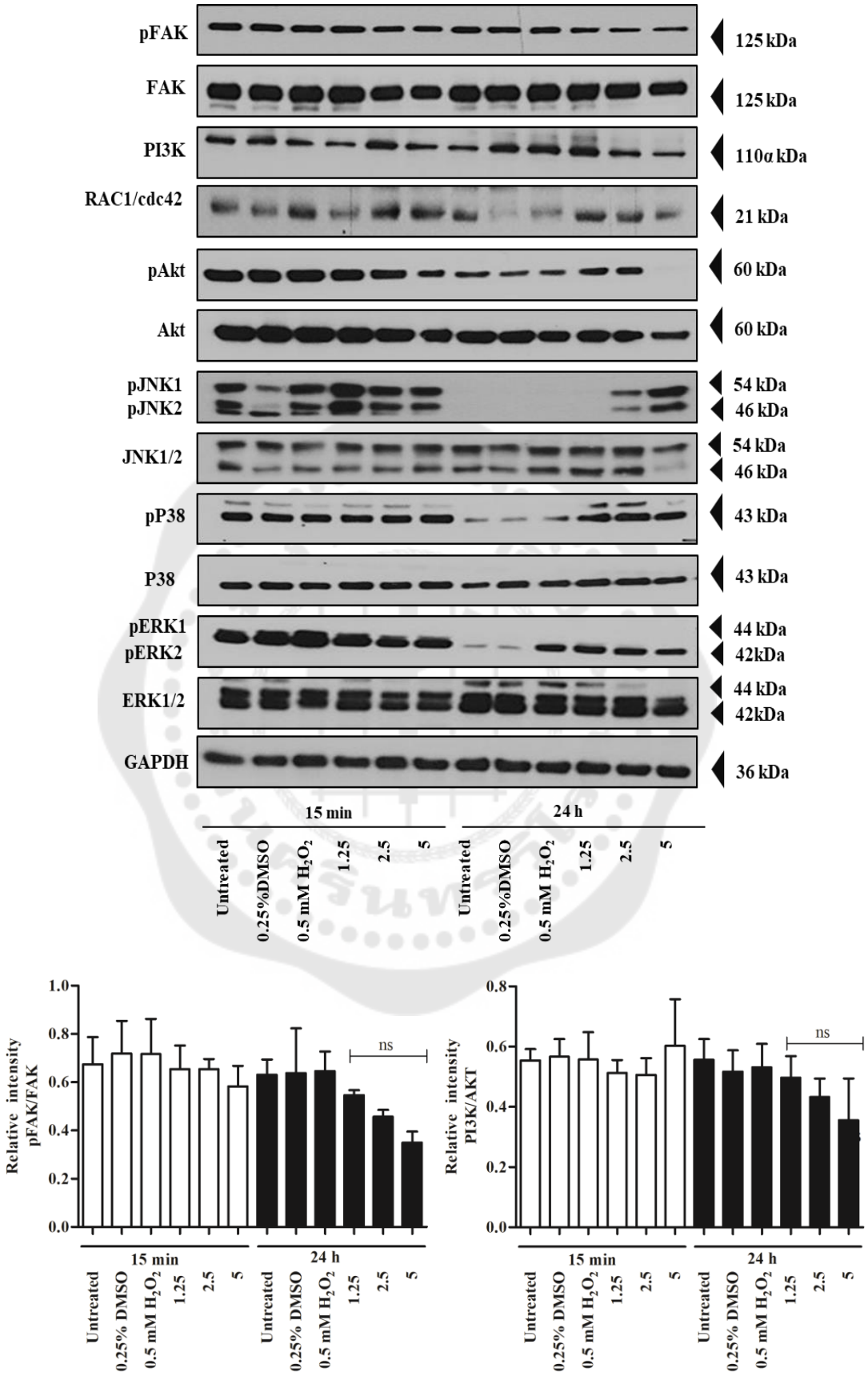


Figure 40 Western blot analysis of MCF-7 treated with 10-HDA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PI3K
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21 kDa
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60 kDa
Akt
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54 kDa
46 kDa
JNK1/2
54 kDa
46 kDa
pP38
43 kDa
P38
43 kDa
pERK1
pERK2
44 kDa
42kDa
ERK1/2
44 kDa
42kDa
GAPDH
36 kDa
15 min
24 h
Untreated
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0.5 mM H2O2
1.25
2.5
5
Relative intensity pFAK/FAK
Relative intensity PI3K/AKT
ns

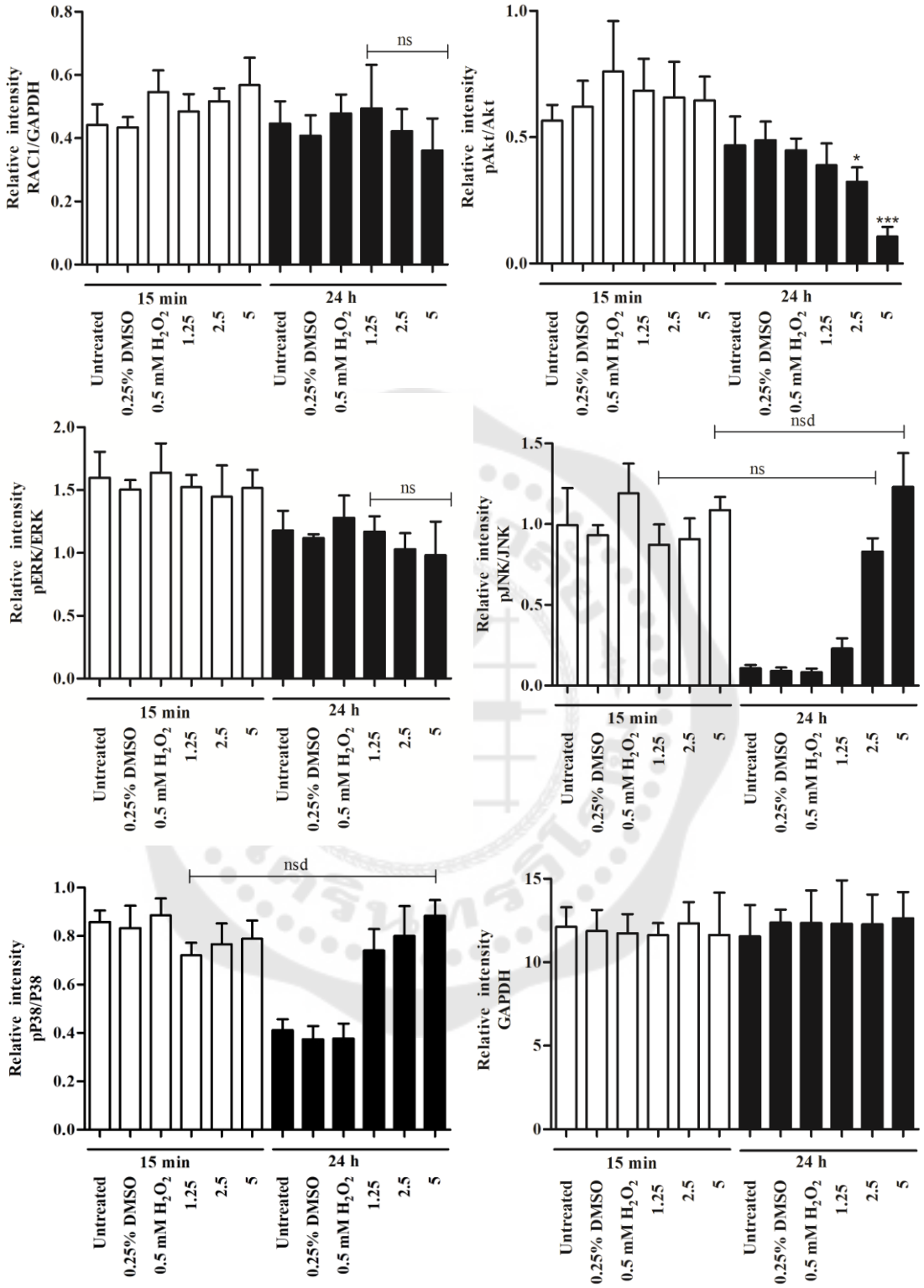
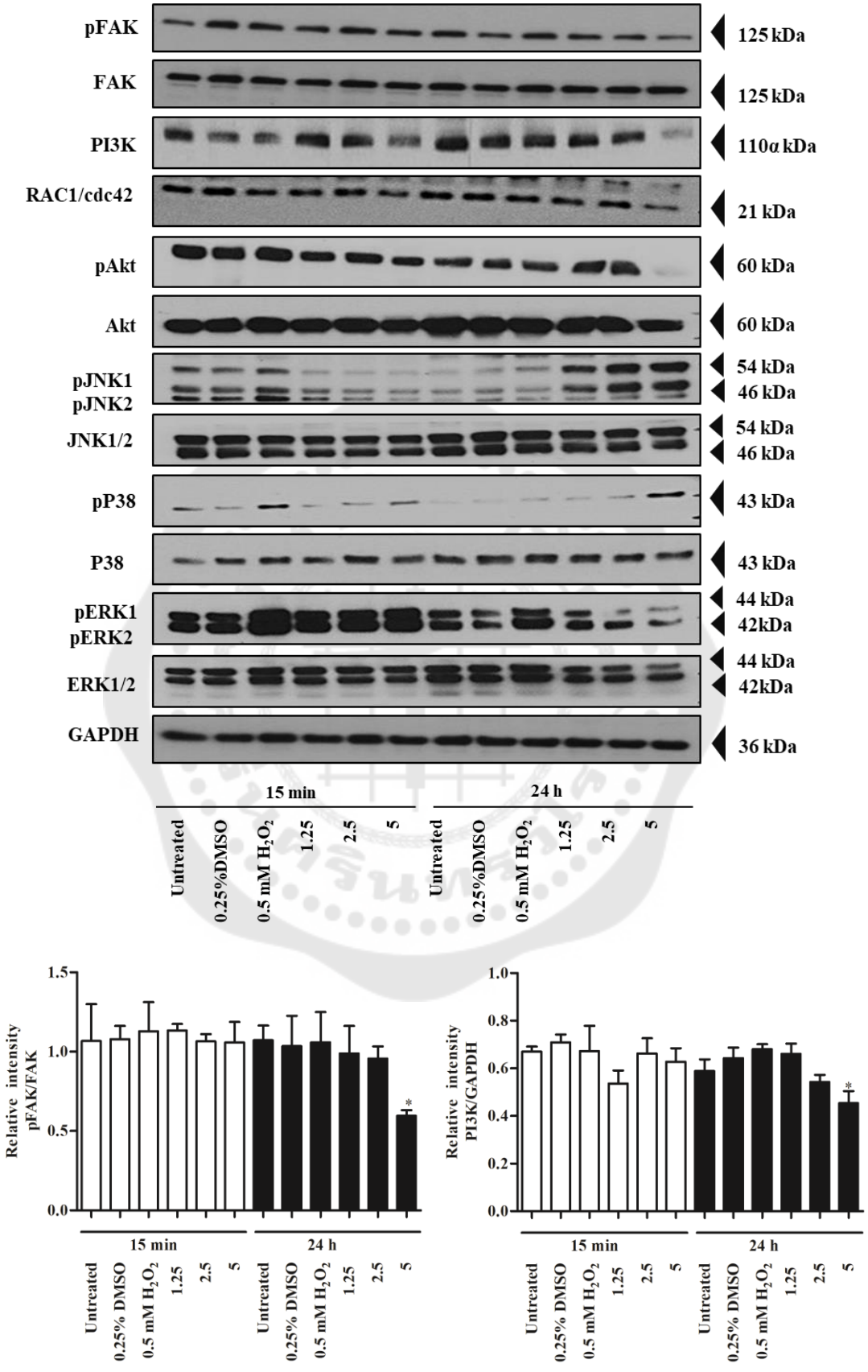


Figure 41 Western blot analysis of NIH3T3 BRAF^{V600E} treated with 10-HDA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PI3K
110α kDa
RAC1/cdc42
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pAkt
60 kDa
Akt
60 kDa
pJNK1
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54 kDa
46 kDa
JNK1/2
54 kDa
46 kDa
pP38
43 kDa
P38
43 kDa
pERK1
pERK2
44 kDa
42kDa
ERK1/2
44 kDa
42kDa
GAPDH
36 kDa
15 min
24 h
Untreated
0.25%DMSO
0.5 mM H2O2
1.25
2.5
5
Relative intensity pFAK/FAK
Relative intensity PI3K/GAPDH
*

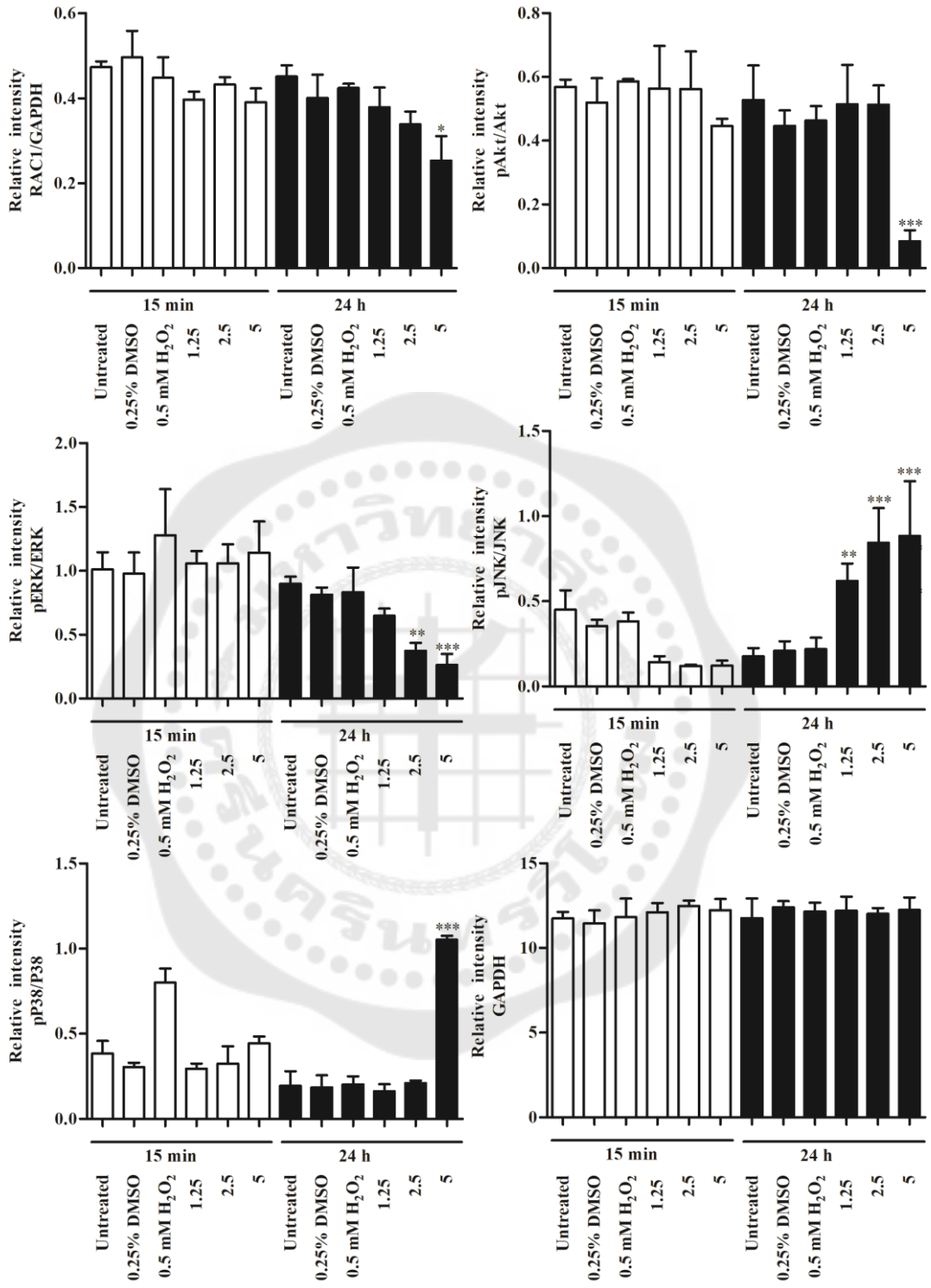
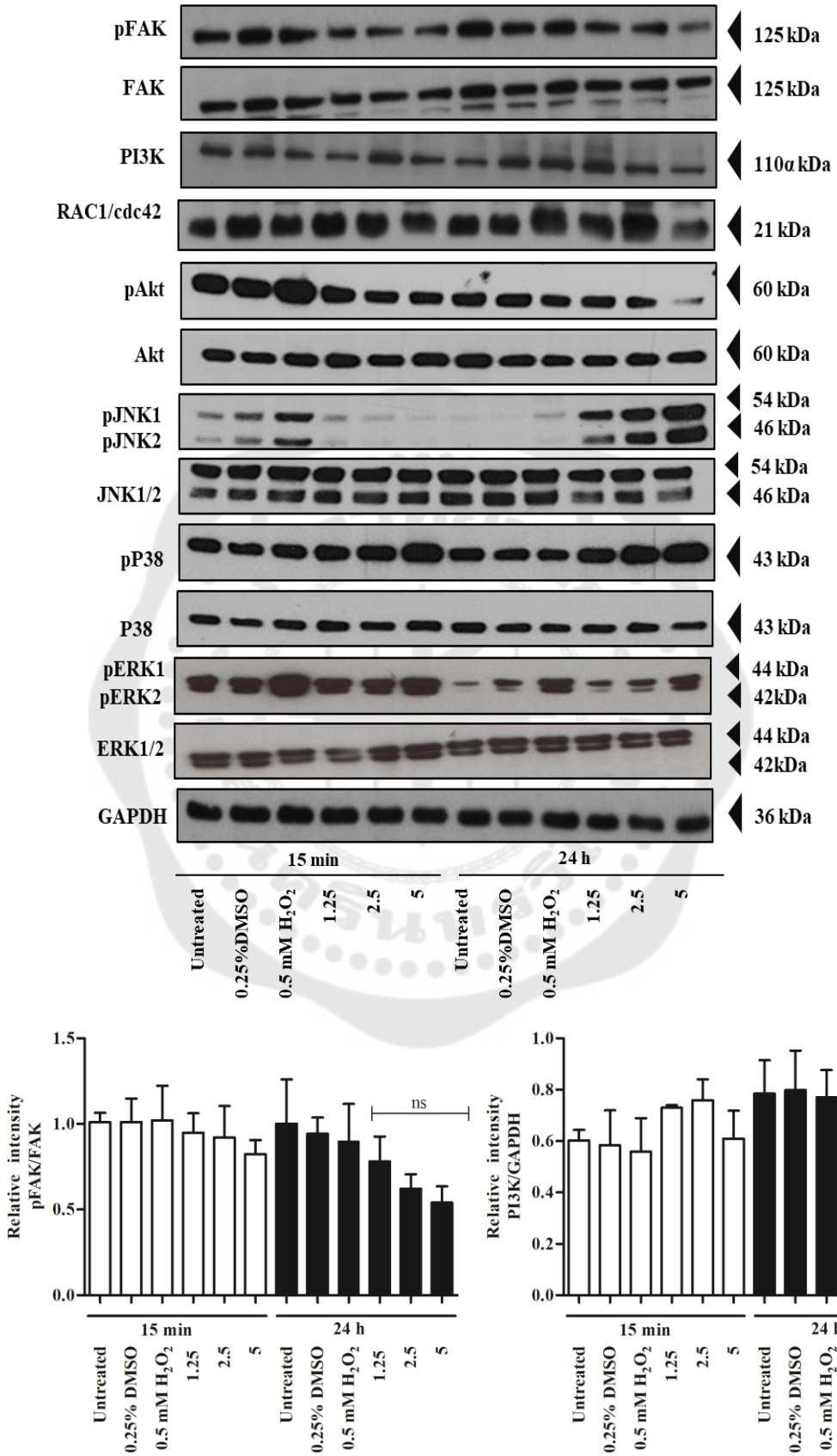


Figure 42 Western blot analysis of MCF-10A treated with 10-HDA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FAK
PI3K
RAC1/cdc42
pAkt
Akt
pJNK1
pJNK2
JNK1/2
pP38
P38
pERK1
pERK2
ERK1/2
GAPDH
125 kDa
125 kDa
110α kDa
21 kDa
60 kDa
60 kDa
54 kDa
46 kDa
54 kDa
46 kDa
43 kDa
43 kDa
44 kDa
42kDa
44 kDa
42kDa
36 kDa
15 min
24 h
Untreated
0.25%DMSO
0.5 mM H2O2
1.25
2.5
5
Relative intensity pFAK/FAK
Relative intensity PI3K/GAPDH
ns

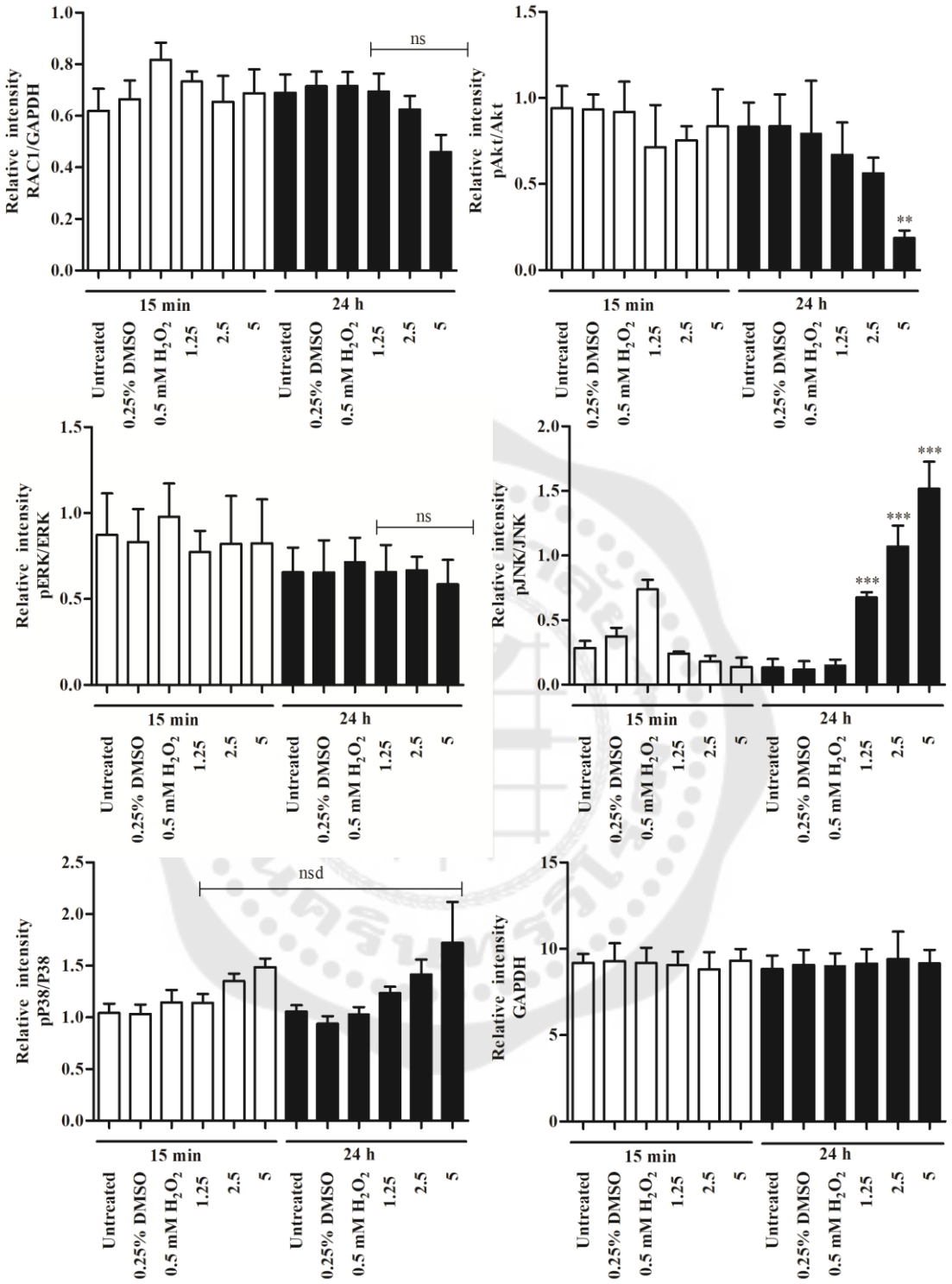

Figure 43 Western blot analysis of NIH3T3 treated with 10-HDA

Table 2 Summary of the signaling pathways of each cell line

Antibody types	pFAK	PI3K	RAC1	AKT	pJNK	pp38	pERK
MDA-MB231	↓	↓	↓	↑	↑	↑	↓
MDA-MB436	↓	↓	↓	↑↑	↑	↑	↓
HCC1937	↓	↓	↓	↑	↑	↑	↓
MCF-7	↓	↓	↓		nsd	nsd	↓
NIH3T3BRAF^{V600E}	↓	↓	↓	↓	↑	↑	↓
MCF-10a	ns	ns	ns	↓	nsd	nsd	ns
NIH3T3 (wt)	ns	ns	ns	↓	↑	nsd	ns

Note: nsd (no significant difference), ns (no significant)

CHAPTER V

DISCUSSION

In the present study, it is firstly demonstrated that 10-HDA has ability to inhibit the proliferation and metastasis of breast cancers. 10-HDA is a major unsaturated fatty acid that existed only in royal jelly, one product of honey bees [131]. Various activities of 10-HDA have been previously reported such as anti-aging, anti-inflammatory, anti-bacterial and melanogenesis inhibitor. Apart from these activities, Izuta et al [5] demonstrated that 10-HDA could inhibit migration, angiogenesis and proliferation in HUVEC. In addition, Filipic et al. 2015 [145] reported that 10-HDA inhibited the proliferation of colorectal adenocarcinoma. So, it is possible that 10-HDA may have an effect on breast cancer cells, especially in the process of metastasis. Triple negative breast cancer cells were selected as models in this study as it has not been studied before. In this report, triple negative breast cancer MDA-MB231 was used as a model of a highly aggressive metastatic breast cancer compared to the other types of triple negative breast cancer MDA-MB436 and HCC1937. MDA-MB231 contains 5 mutations of *BRAF, CDKN2A, KRAS, NF2, P53* and *PDGFRA*. MDA-MB436 has *BRCA1* and *P53* mutations. While, HCC1937 has *BRCA1*, *her2/neu* and *P53* mutations Both MDA-MB231 and MDA-MB436 were derived from the pleural effusion at the metastatic site which do not respond to chemotherapy [157, 158]. Moreover, we also observed in ER positive receptor breast cancer MCF-7 cell line which derived from a pleural effusion taken from a patient with the metastatic breast cancer [159]. Moreover, in this study, we also compared the cytotoxic effect of 10-HDA with other types of normal cell, MCF-10A as a human mammalian epithelial breast cell, Vero cell derived from monkey kidney cell, NIH3T3 received from rat fibroblast and NIH3T3BRAF^{V600E}, a mutant form of NIH3T3 in

which valine (V) is substituted by glutamic acid (E) in *BRAF* gene As BRAF is a proto-oncogene that normally regulate the process of cell growth, so this mutant type has a characteristic of cancer [160].

The cytotoxic effect of 10-HDA was shown as a dose-dependent on a highly metastasis breast cancer MDA-MB231 cells with IC_{50} at 3.5 mM, higher than that of the human mammalian epithelial normal breast control cell MCF-10A, rat fibroblast cell NIH3T3 and kidney monkey cell Vero cells about 1.5, 2.9 and 3.5 folds respectively (Fig 30). While the other types of breast cancer cells were shown the cytotoxic effects similarly to MCF-10A but higher than NIH3T3 and Vero cells about 2 and 2.5 folds, respectively. This data indicates that the cytotoxic of 10-HDA is more specific to breast cancer cells than the control cells. However, the level of cytotoxic in MCF-10A quite similar to breast cancer cells, this is may be due to the genetic of MCF-10A that containing the human telomerase reverse transcriptase (hTERT), making it immortalization and limitless growing. So, this cell line is normally categorized as normal cells but somehow has the property of growth limitless [161].

10-HDA was further investigated on the metastasis processes which play an important role in a stage of cancer and require for the metastasizing of the cancer cell to the distant site. This process includes cell migration, adhesion, invasion and angiogenesis. Additionally, these processes needed to form focal adhesion for mediating the regulation of a cell in response to ECM adhesion. MDA-MB231 was selected as a representative model of TNBC to investigate the effect of 10-HDA on the metastasis of breast cancer. Here, it was shown that 10-HDA significantly inhibited cancer cell migration in a dose-dependent manner which observing from wound healing assay, a semi-quantitative analysis (Fig.31), and transwell migration assay on 2D surfaces (Fig.32). Then, we continued to observe the effect of 10-HDA on the invasion of cancer cells. As it is known that cell invasion is the first step to initiate cell spreading.

This process needs the cells to contact with ECM to form focal adhesion and then, secretes degrading enzymes such as MMPs to degrade ECM components [162, 163]. Transwell invasion was precoated at the upper insert with Matrigel to mimic the ECM and allowing the cancer cells to move through it. As shown in Fig 33, 10-HDA could significantly inhibit the invading ability of MDA-MB231 and seems to be in the same range as affected the migration process. While for the adhesion assay, the array of ECM component proteins; collagen I, collagen II, collagen IV, fibronectin, laminin, tenascin, and vitronectin were used to evaluate the effect of 10-HDA against MDA-MB231. The results demonstrated that 10-HDA inhibited all adhesive molecules which could be observed from the concentration at 0.75 mM (Fig 34). Interestingly, the compound strongly inhibited at some specific molecules which are fibronectin, vitronectin and collagen IV. This information indicates that these three ECM molecules are preferred for breast cancer cell MDA-MB231 to use for adhesion and migration. Especially, fibronectin has been reported that it is one of the extensively characterized molecule that selective binding with $\alpha 5\beta 1$ of integrin [164]. Other works were also shown that these adhesive proteins are crucial for the adhesion property of MDA-MB231 cells [165-167].

To determine further on the effect of 10-HDA on the process of angiogenesis, a key regulator VEGF was selected to be identified. VEGF is a family group of proteins comprises of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF-A is mostly studied and defined as a pre-metastatic niche in the target organs [168]. It is a key regulator of cancer by promoting angiogenesis, cell proliferation, and migration. In addition, it mostly binds and activates the signals via VEGF receptor 2 (VEGFR2) by interaction with integrin alphavbeta3 $(\alpha_v\beta_3)$ [169]. Iso, it can activate FAK and stress-activated protein kinase-2/p38 (SAPK2/p38) to promote cell survival [170]. Interestingly, it has been reported that VEGF-A can mediate the signals without VEGFR2

by directly binding with integrin $\alpha_9\beta_1$ and mediate cell migration and invasion [169]. Here, we found that 10-HDA significantly decreased the releasing of VEGF-A from MDA-MB231 cells implying that the angiogenic ability of breast cancer cells was inhibited (Fig 35). This similar effect has been reported in other work, which 500 μM of 10-HDA suppressed cell migration, proliferation and tube formation in HUVECs [5].

To initiate intracellular signaling of cancer cell migration, ECM molecules selectively bind to the integrin receptors on the cellular membrane and form a special structure called focal adhesions (FAs). This structure serves as a mechanical link between intracellular actin cytoskeleton and several adaptor proteins such as talin, paxillin, and vinculin. This protein assembly allows the growing of actin cytoskeleton networks and drives the extension of flat membrane protrusions called lamellipodia and finger-like protrusions called filopodia to push the membrane forward [171]. The inhibition of these protein assemblies would result in the blockage of the migration property. Therefore, as 10-HDA could inhibit breast cancer cell migration, then we hypothesized that the compound should inhibit the interaction of the actin cytoskeleton and its adaptor proteins. As shown in the co-localization of vinculin and actin, which is a marker of focal adhesion complex, it was seem to be inhibited in MDA-MB231 after treatment with 2.5 mM of 10-HDA for 24 h (Fig 36a). These results were confirmed in MDA-MB436, another type of TNBC (Fig. 36b). Also, we found that the signals of the co-localization reduced in the untransformed epithelial breast MCF-10A cells (Fig. 36c). This data indicates that 10-HDA could inhibit focal adhesion formation both in cancer and normal epithelial breast cells. However, the difference in the level of inhibition was not clearly observed from the immunofluorescent images. Then, a new target that could better indicate the ability of 10-HDA as anti-metastasis in TNBCs are needed to investigate.

To this aim, focal adhesion kinase (FAK) is selected as it is one of the most prominent proteins localized in FAs. FAK has a critical role in cancer progressions, such

as proliferation, survival, metastasis and angiogenesis. Typically, the activation of FAK is induced by integrin clustering upon ECM engagement. The cytoplasmic domain of integrin can interact with FERM domain of FAK and induce a conformational change to allow FAK autophosphorylation at Tyr 397 [172]. Overexpression of FAK is proposed to be a marker for invasive and metastasis in many types of advanced cancers such as breast, ovary, cervical, kidney, lung etc [173]. In breast cancer cells, FAK has been found to be up-regulated not only in the metastatic type but also in DCIS which indicates the importance of FAK as initiating of tumorigenesis [174]. Losing of FAK demonstrated the disruption of microtubules and defective in the formation of focal adhesion [175, 176]. Hence, FAK is seems to be one important target for better control of breast cancer progression. As it is known that FAK is an upstream regulator of PI3-K and RAC1 to control cell migration and also associates with MAPKs to control cell growth and survival. Additionally, the downstream effector of PI3-K is not only RAC1 but also Akt as a close partner. PI3-K/Akt pathway is frequently observed in human cancers, leading to cancer cell proliferation, migration and evade apoptosis even though it's not always in the case. To explore the action of 10-HDA in this aspect, three types of aggressive TNBC (MDA-MB231, MDA-MB436 and HCC1937) have been selected as models for studying. Also, an ER-positive breast cancer (MCF-7) and the human normal breast epithelial cells (MCF10A), rat fibroblast NIH3T3 (wt) and NIH3T3BRAF^{V600E} (mutant) were chosen in order to compare the ability of 10-HDA action. The results showed that the phosphorylated form of FAK significantly reduced upon treatment with 10-HDA at 24 h in all TNBCs. The effect was also observed in MCF-7 cells and NIH3T3BRAF^{V600E} but in a lesser extent. While MCF-10A cells and NIH3T3 cells were shown no effect to FAK phosphorylation.

Due to the phosphorylation of FAK was inhibited, so its downstream effectors are postulated to be affected. To study on the signaling pathway of cell migration, PI3-K

and RAC1 as downstream effectors of FAK were investigated. The result showed that the signal transduction of FAK/PI3-K/RAC1pathway was decreased in a dose-dependent manners and clearly shown in TNBCs and lesser observed in MCF-7 and NIH3T3BRAF^{V600E}, while MCF-10A and NIH3T3 were shown slightly decreased of the signaling. Thus, this result indicated that 10-HDA has the inhibitory effect on cell migration via FAK/PI3-K/RAC1 pathway and rather specifically on TNBCs. In addition, it has been reported that FAK can also be activated RAS, a family of GTPases and identified as an upstream effector of RAF-MEK-ERK pathway, by using adaptor protein GRB2 on its RTK. The activation of RAS can directly activate the catalytic p110α PI3-K subunit (p110 independent to p85), leading to cell proliferation, migration and anti-apoptosis [(177)]. Moreover, FAK has also been directly regulated P38 MAPK and JNK to control cell cycle and apoptosis [(178)]. Overexpression of FAK in cancer has been shown to inhibit MAPKs activation, leading to increase cell proliferation and survival [(179)].

MAPKs (ERK, P38 MAPK, and JNK) were then determined and the result showed that 10-HDA could significantly decrease the phosphorylation of ERK, which demonstrated in all types of breast cancer cells and also the mutant form of BRAF^{V600E} in NIH3T3 but did not effect on two types of control cells. While the phosphorylation of P38 MAPK and JNK was increased in long-term period of incubation in all types of cell samples either cancer cells or normal cells. As we knew that P38 MAPK and JNK are mediators of apoptosis, so the increasing level of them could explain the possibility of apoptosis induction from the effect of 10-HDA. In addition, ERK is normally controlling the process of cell proliferation and will be inhibited under apoptosis induction. So, the decreasing of ERK in this study then supported the hypothesis that 10-HDA may induce apoptosis in breast cancer cells. However, the level of P38 MAPK and JNK were also increased in the control normal cell types, this may explain that 10-HDA has a little

cytotoxic effect of the normal cell types as shown in MTT assay and 10-HDA may induce the cellular stress which leads to respond of JNK and P38 MAPK.

Akt is the downstream effector of PI3-K and frequently act as a close partner in the intracellular signaling to regulate growth and anti-apoptosis. Surprisingly, this study found that PI3-K and Akt was not a closed partner. Akt phosphorylation was significantly increased by a long-term period of 24 h incubation with dose-dependent manners of 10-HDA, while the level of PI3-K was reduced. Additionally, this result of Akt demonstrated only in breast cancer types, but not for MCF-10A, NIH3T3 and NIH3T3BRAF^{V600E}. Overexpression of Akt by 10-HDA treatment in breast cancer cells may possibly that 1) MDA-MB436 is depleted of PTEN which normally function as a natural inhibitor of PI3K/Akt, so making the increasing level of Akt $^{(180)}$. In addition, overexpression of Akt was found in MDA-MB436 and HCC1937 starting from 15 min of incubation implying that it was a fast response compared to other mediators. 2) The mutation of catalytic p110α PI3-K subunit leads to Akt overexpression, which is frequently found in MCF-7. However, the higher level of Akt is normally related to cell survival and proliferation, this data seems to be in the opposite direction as the overall results of breast cancer cells death. This can be explained that Akt may promote cell death instead of cell survival. It has been shown that Akt could inhibit FoxO, a transcription factor that normally upregulates the expression of anti-oxidant proteins. Therefore, the overexpression of Akt would increase in the level of ROS which causing cells undergo apoptosis. The effect of 10-HDA as an apoptosis inducer are consistent with the previously reported of Filipic et al. (2015) reporting that 10-HDA act as oxidative stress induced and consequently induced apoptosis in CaCO2 cells.

In conclusion, this study is firstly demonstrated that 10-HDA has the ability to inhibit cell proliferation and metastasis processes, also inhibit pro-angiogenic VEGF-A induces angiogenesis. Interestingly, 10-HDA could inhibit breast cancer cells in several

pathways via the inhibiting on the phosphorylation of FAK and subsequent downstream effectors which directly affected on cell migration and proliferation via PI3K/RAC1 and ERK pathways. Additionally, the phosphorylation on P38 and JNK MAPK pathway were increased which may be possible that related to stress response induced and followed by cell death as shown in the Fig 44, and also tends to be an oxidative-induced substance. However, further experiments need to be confirming this possibility and hopefully that it will be a good one compound to treat breast cancer. Therefore, these data support the understanding that 10-HDA is an interesting compound to study on the mechanism of cancer metastasis.

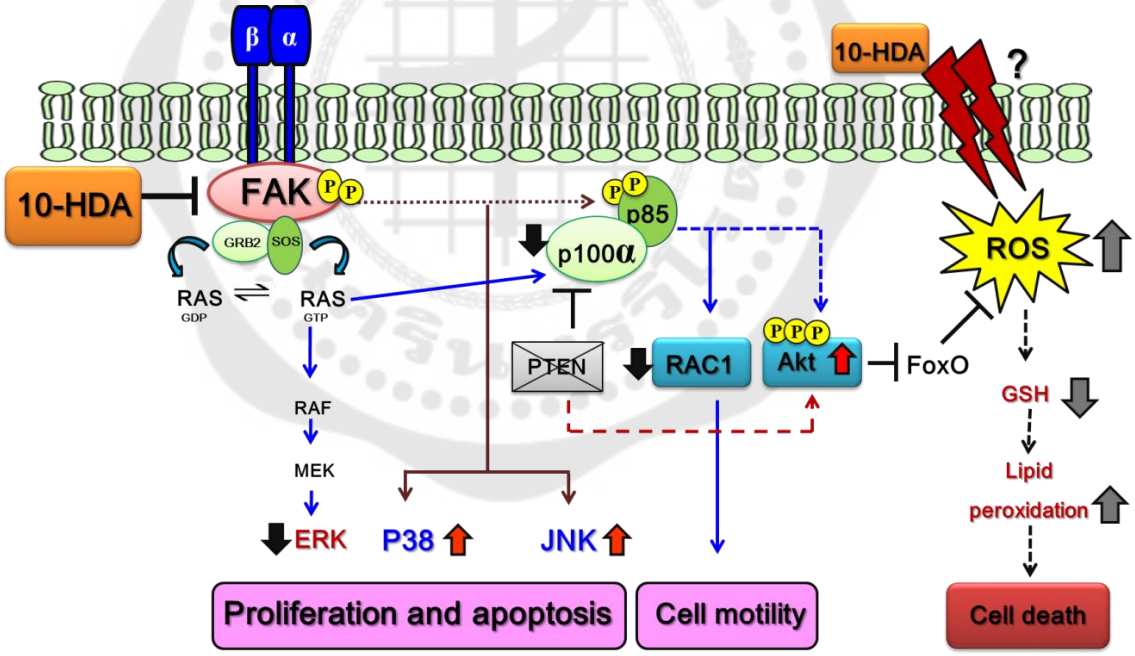

Figure 44 Summary of FAK signaling pathway and its downstream targets under treatment with 10-HDA

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APPENDIX A

REAGENT

MTT solution (for examining cytotoxicity to cancer cells)

Preparation of MTT (0.5 mg/ml)	
MTT	5 mg
Add the culture medium to	10 ml

Cell stain solution (for migration and invasion assays)

Crystal violet	500 mg
Methanol	25 ml
dH_2O	75 ml

Coating solution

50 µg/ml of fibronectin	
0.2 mg/ml of gelatin	
Add MiliQ water to final volume	100 ml

Fixation

4% paraformaldehyde in 1x PBS	100 ml

Permeabilization

0.1% Triton X-100 in 1x PBS	100 ml

1X wash buffer

1X PBS	100 ml
0.5% Tween 20	5 µl

Blocking solution

1% BSA in 1x PBS	100 ml

APPENDIX B

BUFFER

TE buffer

1M Tris, pH 8.0	10 ml
0.5M EDTA, pH 8.0	2 ml
H_2O to 1000 ml	

NP40

25 mM TRIZMA base

150 mM NaCl

10 mM $Na_4P_2O_7$

25 mM β-glycero-phosphate

10% glycerol

0.75% NP-40,

25 mM NaF

pH 7.2

Na_3VO_4

Lysis buffer

NP40	1 ml
Proteinase inhibitor	10 µl

1 M TrisHCl pH 6.8

Trishydroxymethylaminomethan (trizma)	12.11 g

Add MilliQ water up to 100 ml

Adjust pH with HCl to 6.8

Filter (0.45 µm), store at 4°C, use at room temperature

1 M Tris HCl pH 8.8

Trishydroxymethylaminomethan	60.55 g
Add MilliQ water up to 500 ml	
Adjust pH with HCl to 8.8	
Filter (0.45 µm), store at 4°C, use at room temperature	

20% SDS

Sodiumdodecylsulfate	20 mg
Add MilliQ water up to 100 ml	
Filter (0.45 µm), store at room temperature	

10% APS

Ammoniumpersulfate	100 mg
Add 1 ml MilliQ water	
Store at 4°C (1 week) or – 20°C (1 month)	

10x Running buffer

Trishydroxymethylaminomethan (TRIZMA)	30 g
Glycine	144 g
SDS	10 g
Add MilliQ water up to 1000 ml	
pH = 8.3 (unadjusted), store at room temperature	

1x Running buffer

10 x Running buffer	100 ml
MilliQ water	900 ml

6x Laemmli

1 M TrisHCl pH 6.8	12 ml
SDS	12 g

0.5 M.EDTA pH 8.0	2.4 ml
Glycerol	20 g
Bromphenalblue	100 mg
Add MilliQ water up to 100 ml	
Mercaptoethanol	4.75 ml

10 x Blotting buffer

250 mM TRIZMA Base	30.3 g
1920 mM Glycine	144 g
0.1% SDS	1 g
Add MilliQ water up to 1000 ml (pH 8.5 unadjusted)	

1x Blotting buffer

10x Blotting buffer	100 ml
20% Methanol	200 ml
Add MilliQ water up to 1000 ml	
Store at 4°C	

10x TBS

500 mM TRIZMA Base	60.55 g
1500 mM NaCl	87.66 g
Add MilliQ water up to 1000 ml	
pH 7.5 with HCl	

1x TBST

10 x TBS	100 ml
0.1% Tween 20	1 ml
Add MilliQ water up to 1000 ml	

5% TBSTM

Milk Powder	2.5 g
1x TBST	50 ml
Use it fresh	

Stripping buffer

200 mM Glycine, pH=2.5	15 g
0.05 Tween 20	500 µl
100 mM 2-Mercaptoethanol	7.8 ml
H_2O to 1000 ml	

Phosphate buffered saline (1XPBS)

For preparation of the solution, all compounds in the following order dissolve in distilled water.

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
H_2O (distilled water)	800 ml
H_2O (distilled water) adjust volume to 1000 ml	
Adjust pH 7.4 with HCl	

The solution was sterilized by 0.2 µm filter membrane and stored at room temperature.

APPENDIX C

CULTURE MEDIUM

M199 medium (for Vero cell)

M199 medium (Gibco RRL, NY, USA)	1 pack
$NaHCO_3$	2 g
Sterile water to make	1000 ml
Adjust pH 7.2 with HCl or NaOH before adjusting volume with water and sterile by cellulose acetate filter (pore size 0.22 µm)	
Penicillin (100 U/ml) / streptomycin (100 µg/ml) with the ratio 1:100	
10% FBS	

DMEM medium (for MDA-MB231 NIH3T3 (wt) and NIH3T3 BRAFV600E) 500 ml

DMEM medium solution, Lonza, USA	5 ml
200 mM L-glutamine	5 ml
Penicillin (100 U/ml) / streptomycin (100 µg/ml) with the ratio 1:100	50 ml
10% FBS	

DMEM medium (for MDA-MB436)

DMEM medium solution, Lonza, USA	500 ml
200 mM L-glutamine	5 ml
Penicillin (100 U/ml) / streptomycin (100 µg/ml)	5 ml
10% FBS	50 ml
10 µg/ml ITS (insulin-transferrin-sodium selenite)	
16 µg/ml L-glutathione	

RPMI1640 medium (for HCC1937)

RPMI1640 medium solution, Lonza, USA	500 ml
200 mM L-glutamine	5 ml
Penicillin (100 U/ml) / streptomycin (100 µg/ml)	5 ml
10% FBS	50 ml

MEM medium (for MCF-7)

MEM medium solution, Sigma-Aldrich, Germany	500 ml
200 mM L-glutamine	5 ml
Penicillin (100 U/ml) / streptomycin (100 µg/ml)	5 ml
10% FBS	50 ml

DMEM/F12 (1:1 v/v) medium (for MCF-10a)

DMEM/F12 (1:1 v/v) medium, Lonza, USA	500 ml
200 mM L-glutamine	5 ml
Penicillin (100 U/ml) / streptomycin (100 µg/ml)	5 ml
5% FBS	50 ml
EGF 20 ng/ml	
Hydrocortisone 0.5 µg/µl	
ITS (insulin-transferrin-sodium selenite) 10 µg/ml	

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