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Identifying Novel Metastatic Cell Lines and Exploring ITCH's Role in Invadopodia Formation

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

Ann Truong

Committee in charge:

Professor Jing Yang, Chair
Professor Dong-Er Zhang, Co-Chair
Professor Gen-Sheng Feng

2024

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University of California San Diego

2024

DEDICATION

This thesis is dedicated to my parents, whose unwavering support, encouragement, and sacrifices have enabled me to achieve more than we ever imagined. Their belief in me, even without fully understanding my studies, has been invaluable and something I appreciate deeply. I also dedicate this work to my close friends, whose unconditional support and understanding have kept me motivated throughout this arduous journey.

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LIST OF ABBREVIATIONS

DMSO: Dimethyl sulfoxide

ECM: Extracellular matrix

EGF: Epidermal growth factor

EMT: Epithelial-to-mesenchymal transition

OG: Oregon-green

PFA: Paraformaldehyde

SFM: Serum-free medium

shRNA: Short hairpin RNA

Tks5: Tyrosine kinase substrate 5

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Additionally, I would like to extend my thanks to every single one of the wonderful members in the Yang Lab. Your willingness to help me at every step, whether that be related to science or to outside life, has been instrumental to my time here, and I will never forget it.

ABSTRACT OF THE THESIS

Identifying Novel Metastatic Cell Lines and Exploring ITCH's Role in Invadopodia Formation

by

Ann Truong

Master of Science in Biology

University of California San Diego, 2024

Professor Jing Yang, Chair
Professor Dong-Er Zhang, Co-Chair

Metastasis is a complex process in which cancer cells spread from a primary tumor site to invade surrounding tissue, enter the bloodstream, and propagate into distant organs. One of the methods in which metastasis occurs is through Epithelial-to-Mesenchymal Transition, where the extracellular matrix is degraded and restructured; consequently, this enhances cellular migration,

invasion, and ultimately, metastasis. A key component of this process is the formation of actin-rich protrusions called invadopodia that serve to break down the extracellular matrix, thereby facilitating cancer invasion. Meanwhile, the E3 ubiquitin ligase ITCH, a key regulator in ubiquitination, has an unexplored role in invadopodia formation. My project thus aims to elucidate ITCH's contribution to tumor invasion via invadopodia-mediated degradation across various cancer cell lines.

I initially focused on ITCH's role in invadopodia-mediated degradation in the MDA-MB-435 (melanoma) and MDA-MB-231 (triple-negative breast cancer) cell lines, whose ability to form invadopodia was unknown. Degradation and immunofluorescence assays were conducted at various time points and cell confluences. Based on these results, the analysis was expanded to more aggressive cell lines: A673 (Ewing sarcoma), EWS-502 (Ewing sarcoma), TC-32 (neuroectodermal), and SK-N-MC (neuroblastoma). Degradation assays were repeated at different time points and concentrations. Promising results led to further investigation of A673 and EWS-502 by downregulating ITCH expression via lentivirus-mediated shRNA gene knockdown and repeating the degradation assays. Despite comprehensive analysis, the results were inconclusive. However, this project provided preliminary data and established a foundation for future studies to determine the precise role of ITCH in invadopodia formation and degradation, particularly in the A673 and EWS-502 cell lines.

INTRODUCTION

Metastasis

By definition, metastasis is the highly selective process by which cancer cells disseminate from an original, primary tumor site to distant, secondary sites in the body. There, these cells attach and form new tumors. Unlike primary tumors that can often be surgically removed or locally treated through radiation, tumors that have metastasized prove to be a difficult challenge due to their distant spread and therefore cannot be treated the same way. Complete eradication of all tumor cells is needed to prevent recurrence, and partial destruction could adversely lead to even more rapid cellular proliferation, enhancing heterogeneity and overall tumor resistance (Siegel et al., 2024). Heterogeneity refers to the different biological properties of specific cancer cells within the same tumor that individually exhibit distinct genetic, phenotypic, and behavioral traits (Fidler, 1989); this level of specificity requires a plethora of adaptive and personalized treatment strategies to effectively combat metastatic cancer growth.

Meanwhile, even though metastatic progression at the molecular level is well-studied, the exact mechanistic explanation behind how and why it occurs is still being explored. During this metastatic process, otherwise known as the metastatic cascade, cancer cells initially detach from the primary tumor and are most commonly transported via the circulatory and lymphatic system, where they must survive general immune system defenses. If they ultimately survive, they then move forward to adhere to capillary endothelial cells and other lymphatic structures, thus allowing them to extravasate into the core essential functional tissue of the distant organ. Once the secondary tumors at these organs reach a certain size, further metastatic lesions can form, spreading even further throughout the body (Fidler, 1989).

Epithelial-to-Mesenchymal Transition (EMT)

Epithelial cells typically form organized layers with closely connected cells using specialized membrane structures, which include but are not limited to tight junctions, adherens junctions, desmosomes, and more. They also exhibit apical-basolateral polarization and are normally only motile within the epithelial layer where they do not detach and move away. On the other hand, mesenchymal cells lack cell layers, apical-basolateral polarization, and continuous junctions; they only connect at specific points and continue to grow into spindle-shaped cells that are highly motile (Thiery & Sleeman, 2006). Epithelial-to-Mesenchymal Transition (EMT) is then the process by which epithelial cells gradually lose their stable characteristics and gain more mobile mesenchymal-like qualities; they progressively redistribute or downregulate their tight junction proteins, which include E-cadherin and cytokeratins, and re-express new molecules commonly seen in mesenchymal cells, such as vimentin and N-cadherin (Christofori, 2006). This change increases the efficacy of cellular invasion into neighboring tissues through enhanced migratory and intrusive abilities (Geiger & Peeper, 2009) (Figure I.1).

Even though this molecular switch is considered a key player in cancer progression and metastasis, its exact mechanistic role in tumor progression is still not completely understood. However, in other contexts, EMT activation has been studied to be involved in many embryonic processes, such as gastrulation, neural crest formation, heart valve formation, and wound healing (Thiery & Sleeman, 2006).

Specifically in tumor cells, EMT activation can be triggered by a wide variety of factors. Some of these activating signals come from the tumor microenvironment, like Wnt, Notch, and growth factor receptor signals. Some key growth factors include transforming growth factor- β (TGF- β), hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth

factors (IGFs) and fibroblast growth factors (FGFs); upregulated proteolytic activity of matrix metalloproteases (MMPs) was also observed in EMT induction (Christofori, 2006). Other activating signals include inflammatory cytokines, hypoxia, and changes in extracellular matrix density and rigidity (Fontana et al., 2024). These signaling responses then trigger the activation of intracellular effector molecules, including members of the small GTPase family and members of the Src tyrosine-kinase family. These effectors work by coordinating the breakdown of cell junctions and the reorganization of the cell's structure that happens during EMT. Activating these signaling pathways also turns on transcriptional regulators like snail (SNAIL) and slug (SNAI2), which control the changes in gene expression that drive EMT (Thiery & Sleeman, 2006).

The complex aspects regarding the nature of EMT additionally serve a role in promoting cellular metastasis in a multitude of ways. Due to reduced cell adhesion, tumor cells are generally able to move and invade tissues and vessels by undertaking motile mesenchymal characteristics. Furthermore, cells undergoing EMT commonly secrete matrix metalloproteinases (MMPs) which act by remodeling the extracellular matrix (ECM), thereby releasing and processing growth and blood vessel-forming factors, while also exposing sites that promote cell migration and angiogenesis. EMT can also disrupt essential intracellular signaling, such as with E-cadherin and its corresponding receptors in various pathways, induce invasive mesenchymal proteins (such as N-cadherin), and inhibit cellular apoptosis (Geiger & Peeper, 2009).

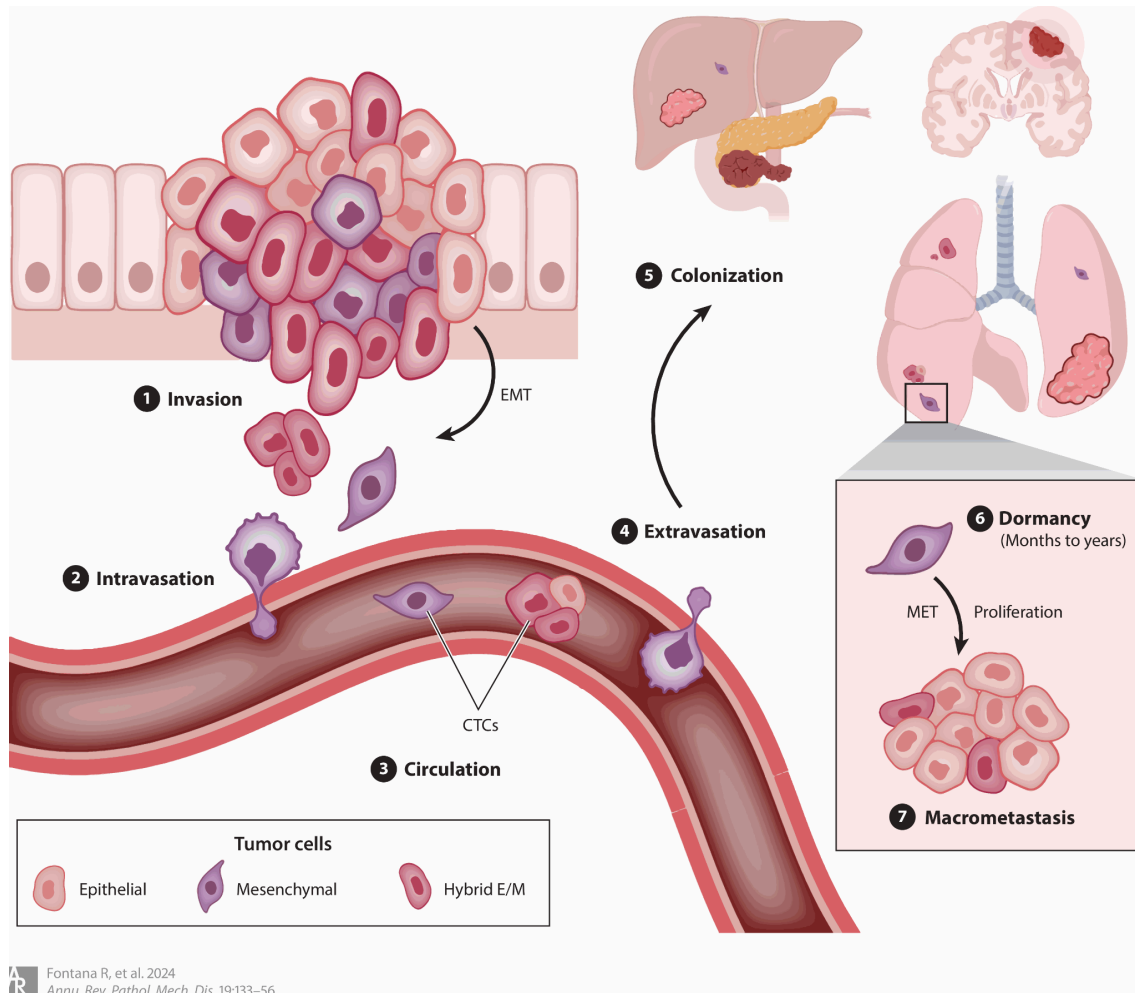


Figure I.1: EMT in tumor progression and metastasis. Adapted from Fontana R., et al. 2024.

Invadopodia

Invadopodia are small, finger-like projections that puncture into the extracellular matrix (ECM) and are filled with actin. They are generally formed by motile, mesenchymal-like cells, including various cancer cells and other stimulated endothelial cells (Weaver, 2006) (Figure I.2). These structures have additionally been shown to be essential in the degradation of the ECM during a variety of cellular processes, such as morphogenesis, differentiation, cell migration,

apoptosis, neo-angiogenesis, and in metastatic cell behavior (Ayala et al., 2006). In addition, they are uniquely not present in normal cells.

At the structural level, invadopodia engage a large number of relevant proteins that contribute to its development. Cortactin, a cytoskeletal protein, and MENA, a member of the Enabled (Ena)/VASP family, are key regulators of actin polymerization, which support invadopodia formation. In addition, Tks4 and Tks5 are adaptor proteins shown to be critical for protein and lipid protein for invadopodia assembly. Due to its degrading properties, invadopodia also contain a large number of proteases; these include MT1-MMP (a metalloproteinase involved in substrate cleavage), ADAM12 (an integrin modulator), and other serine proteases, such as FAP and DPP4. Due to its invasive nature, invadopodia-mediated matrix degradation is a highly regulated process. Several of its regulatory mechanisms involve the Src kinase, tyrosine kinases, key integrins, and additional EMT-inducing transcription factors, such as Twist1 and TGF β . All of these components therefore collectively play a fundamental role in invadopodia growth and expansion (Paz et al., 2014).

Based on this understanding, the assembly of invadopodia involves several stages. Initially, polarization and Src (a family of non-receptor tyrosine kinases) signaling play a critical role. Focal adhesion kinase (FAK) regulates invadopodia by interacting with Src, either promoting or inhibiting their formation. In the next stage, invadopodia assembly is heavily dependent on PI3K signaling, since PI3K activity is essential for invadopodia formation and function by influencing the assembly of actin filaments and the localization of critical proteins. Meanwhile, as mentioned before, Tks4 and Tks5 proteins are also crucial for both the formation and function of invadopodia, with Cortactin playing a key role in regulating actin organization and proteinase trafficking. Finally, the dynamic process of assembly and disassembly of

invadopodia, otherwise referred to as “turnover”, occurs over a time span ranging from minutes to hours. This process is significantly influenced by the tyrosine phosphorylation of cortactin and AFAP110 (Actin Filament Associated Protein 110), which is essential for the proper turnover of these structures (Saykali & El-Sabai, 2014).

Overall, invadopodia structures play an undoubtedly vital role in facilitating cancer cell metastasis. Their abilities to degrade the ECM are crucial for tumor cells to invade surrounding tissue and metastasize; thus, invadopodia formation is correlative with invasive and metastatic potential for tumor cells. Furthermore, they are involved in cellular intravasation into blood vessels as well as extravasation out of these blood vessels to form secondary tumors. As a result, targeting invadopodia formation and function could be a promising strategy to inhibit tumor invasion and metastasis (Yamaguchi, 2012).

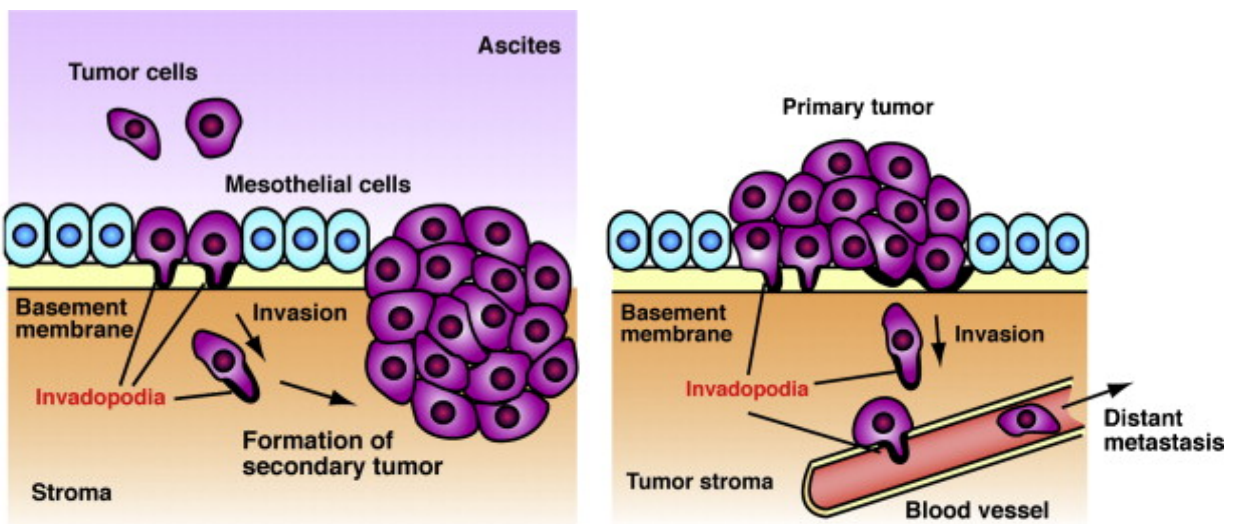


Figure I.2: Schematic models of the roles of invadopodia in cancer metastasis.
 Adapted from Yamaguchi, H., 2012.

Ubiquitination and the E3 Ubiquitin Ligase (ITCH)

Ubiquitination is a post-translational modification process in which ubiquitin, a small regulatory protein, is attached to a substrate protein. This system generally involves three main types of enzymes (Pickart, 2001):

1. E1 (Ubiquitin-Activating Enzyme), which depends on ATP to activate ubiquitin.
2. E2 (Ubiquitin-Conjugating Enzyme), which transfers activated ubiquitin from E1 to the substrate.
3. E3 (Ubiquitin Ligase), which oversees the interplay between substrate recognition and binding, thereby facilitating the transfer of ubiquitin from E2 to the final substrate.

Notably, there are over 600 identified E3 ubiquitin ligases, targeting specific substrate proteins. These ligases are broadly categorized into three general families: the RING finger family, HECT family, and RBR family. The RING finger family contains the RING or U-box catalytic domain, which is rich in cysteine; they function as a mediator between the transfer of ubiquitin from E2 to the substrate without directly binding to the ubiquitin. Meanwhile, the HECT family E3 ligases contain a HECT catalytic domain and a cysteine active site, where the E2 ubiquitin forms an intermediate thioester bond before being transferred to the substrate. Lastly, the RBR family functionally acts as a RING-HECT hybrid (Sampson et al., 2023). ITCH, whose full name is itchy E3 ubiquitin protein ligase, resides in the HECT family.

At the molecular level, ITCH is an E3 ligase whose catalytic activity is stimulated by phosphorylation; the enzyme works by attaching ubiquitin molecules to substrate proteins, thereby marking them for degradation by the proteasome. ITCH has three key phosphorylation sites that are phosphorylated by JNK1. Phosphorylation at these sites disrupts and weakens the

interaction between the WW and HECT domain of ITCH, resulting in enzyme activation (Gallagher et al., 2006).

Studies have shown that E3 ubiquitin ligases play a significant role in various cancer hallmark pathways, particularly in cycle progression, immune evasion and inflammation, and apoptosis (Sampson et al., 2023). Furthermore, ITCH in particular has been found to promote EMT by activating specific proteins that enhance ECM degradation and invasion potential of cancer cells (Salah et al., 2014). Thus, my project is focused on exploring the link between ITCH, invadopodia, and ECM degradation.

CHAPTER 1: PRELIMINARY CELL LINE SCREENING TO INVESTIGATE THE FUNCTION OF ITCH IN METASTASIS

Invadopodia are small, finger-like projections that penetrate the extracellular matrix (ECM). They are uniquely found in cancer cells and have been shown to be essential in ECM degradation. This ability to degrade the ECM is crucial for tumor cells to invade, metastasize, and form secondary tumors, as invadopodia formation is linked to both invasive potential and cellular intravasation and extravasation.

Metastasis occurs when cancer cells proliferate from a primary tumor site to distant organs in the body, usually through the circulatory or lymphatic system. This invasive process is characterized by degradation of the ECM, and studies have shown that invadopodia play a critical role in this process, particularly in several cancer cell lines and primary tumor cells. Some of the previously studied cell lines include the bladder (Yamamoto et al., 2011), colorectal (Schoumacher et al., 2010), breast (Coopman et al., 1998), and squamous carcinoma cells (Takkunen et al., 2010).

This first chapter will address if two new cancer cell lines will display ECM degradation and invadopodia formation; these cell lines are MDA-MB-435 (melanoma) and MDA-MB-231 (highly aggressive triple-negative breast cancer).

Matrix degradation assays for MDA-MB-435 cell line

To evaluate the degradation potential of the MDA-MB-435 cell line, I conducted a 2D-fluorescent matrix degradation assay. This highly sensitive method accurately measures invadopodia-mediated ECM degradation (Fontana & Yang, 2021). In this assay, cells are seeded on coverslips coated with Oregon green (OG) 488-conjugated gelatin matrix that allows for invadopodia formation and pinpoints the location of ECM degradation through the detection of dark spots. Additional staining was performed with phalloidin for filamentous actin (F-actin) to show the cell cytoskeleton and DAPI for the cell nucleus.

The MDA-MB-435 cell line is a well-studied melanoma cell line with a spindle-shaped morphology, and it is reported to be highly metastatic (Meng et al., 2019). To perform the degradation assay, two different numbers of cells were plated on OG-gelatin-coated coverslips in triplicate and incubated for 16 hours and 24 hours. The samples in the 16-hour degradation assay displayed little to no degradation for both cell concentrations: 2×10^4 cells/well and 4×10^4 cells/well. The samples that were incubated for 24 hours displayed slightly more degradation, but not sufficient for quantification (Figure 1.1).

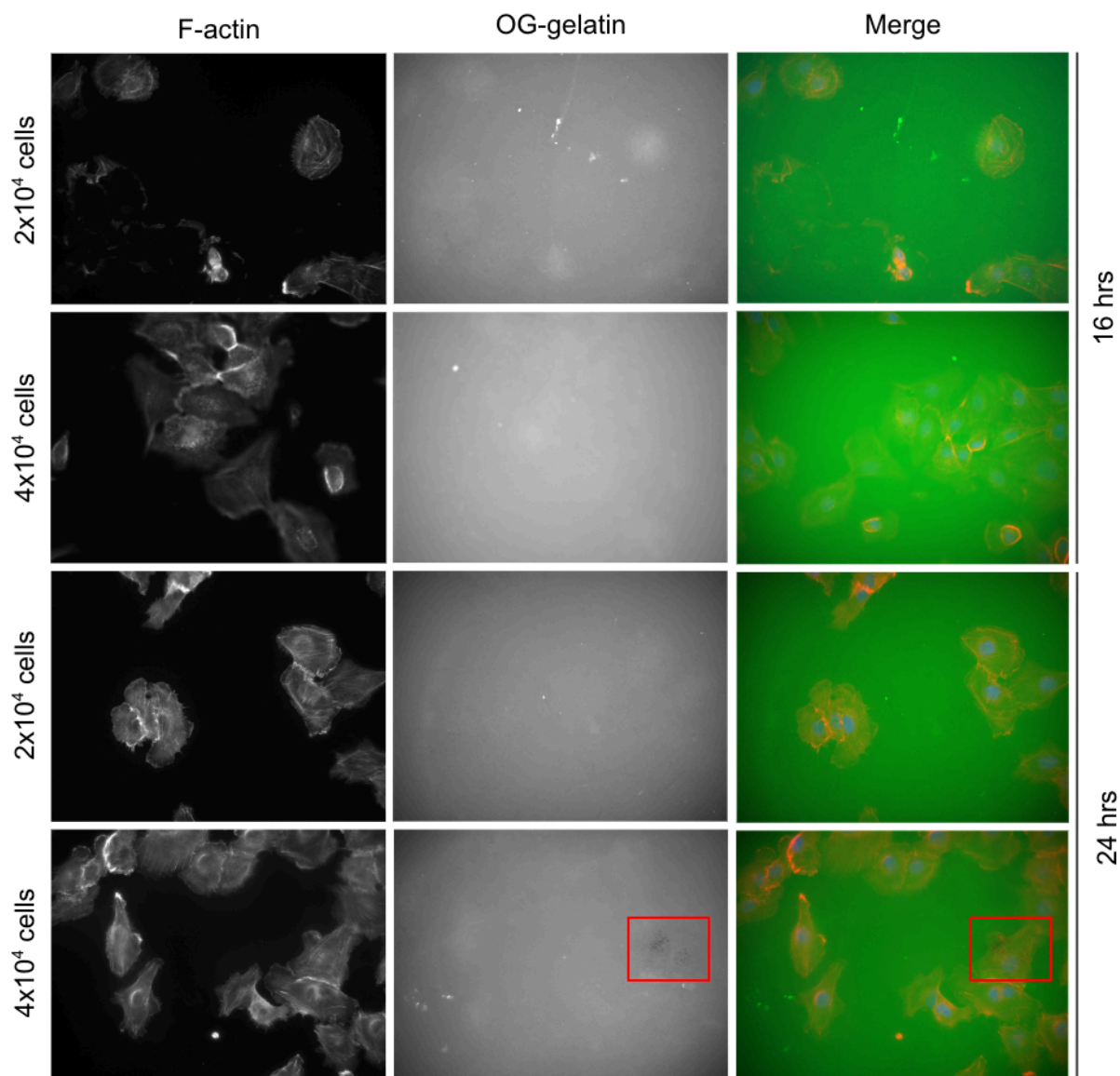


Figure 1.1: Matrix degradation assays for MDA-MB-435 cell line. Cells were either plated with 2×10^4 cells/well or 4×10^4 cells/well. Cells were then incubated on OG-gelatin coated coverslips for 16 and 24 hours. Nuclei were stained with DAPI and are shown in blue. F-actin were stained with phalloidin and are shown in red. OG gelatin is shown in grayscale and areas of degradation appear as dark dots. Grayscale colors are used for better visualization; merged images show degradation co-localization with cell structure.
Note the slight degradation boxed in red.

Following the initial degradation assays, the MDA-MB-435 cell line was plated again to test several additional conditions. Three different concentrations were plated this second time:

0.5×10^4 , 1×10^4 , and 2×10^4 cells/well. The cells were also cultured on OG-gelatin coated coverslips for three different time points: 16 hours, 24 hours, and 48 hours. The cells were fixed and stained with phalloidin and DAPI for F-actin and DNA, respectively (Figure 1.2).

The SCC61 head and neck squamous cell carcinoma cell line was added as a positive control since the SCC61 cell line is known for its ability to form invadopodia and degrade ECM very well (Bertier, et al., 2018); thus, it is used as a positive control in this experiment.

The results of the degradation assay demonstrated that the MDA-MB-435 cell line does not degrade well, especially when compared to the SCC61 positive control cell line.

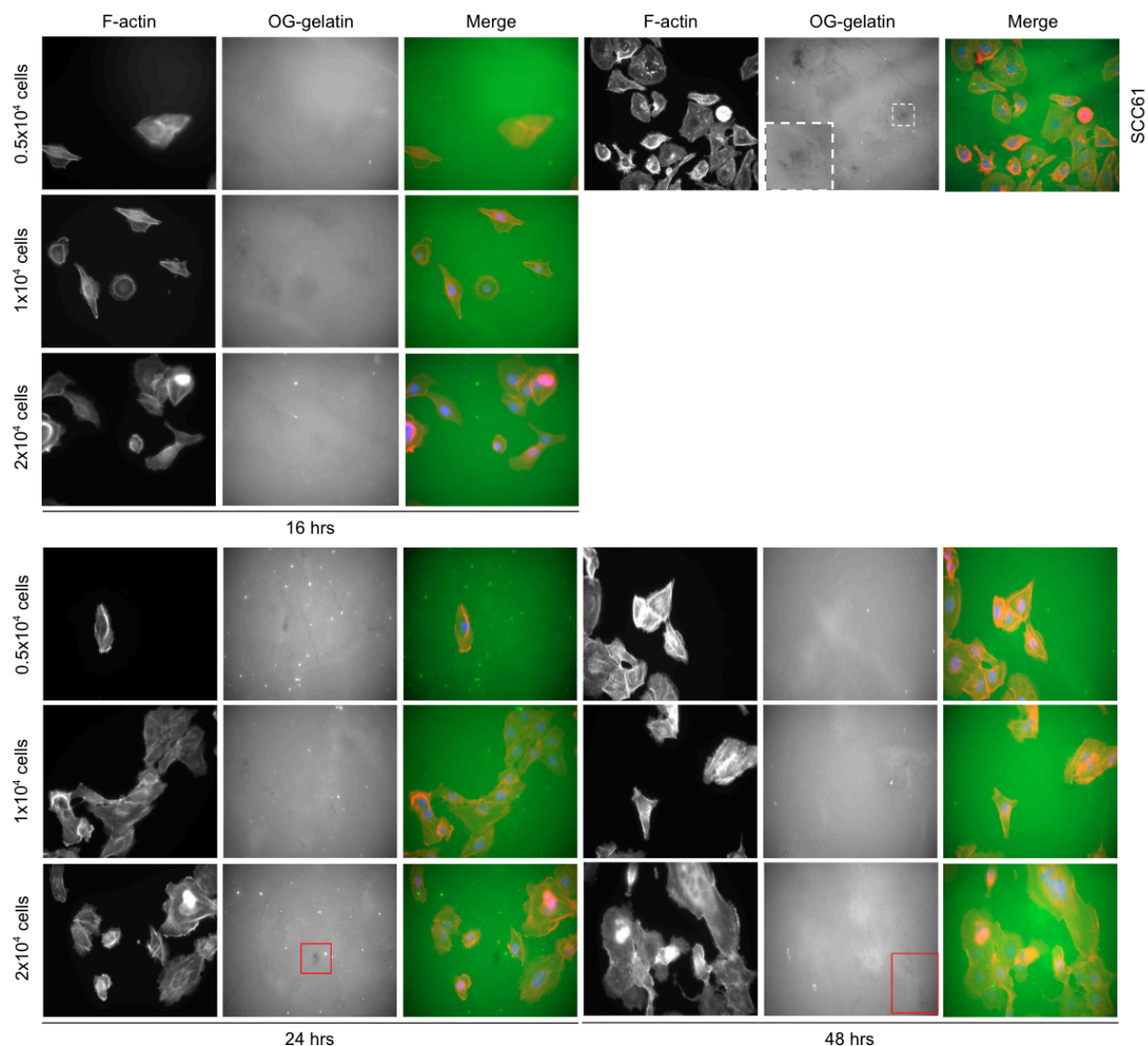


Figure 1.2: Repeated: Matrix degradation assays for MDA-MB-435 cell line. Target cells were plated with 0.5×10^4 , 1×10^4 , or 2×10^4 cells/well. Cells were then incubated on OG-gelatin coated coverslips for 16, 24, or 48 hours. The SCC61 control cells were plated with 2×10^4 cells/well and incubated for 16 hours. Nuclei were stained with DAPI and are shown in blue. F-actin were stained with phalloidin and are shown in red. OG gelatin is shown in grayscale and areas of degradation appear as dark dots. Grayscale colors are used for better visualization; merged images show degradation co-localization with cell structure. Note the slight degradation boxed in red.

Matrix degradation assays for MDA-MB-231 cell lines

The MDA-MB-231 cell line is a widely used metastatic human triple-negative breast cancer cell line (Hegedüs et al., 2008). It is also characterized as being highly invasive and aggressive, therefore holding potential for exhibiting ECM degradation and invadopodia formation. However, prior research using MDA-MA-231 cells obtained from ATCC has not been able to detect degradation, apart from a study done by a group in Oslo, Norway (Pedersen et al., 2021). They found that through a serum-free starvation and degradation re-stimulation protocol, invadopodia formation could be induced. Based on the results of that study, we were able to obtain a vial of their cells. Moving forward, these MDA-MA-231 cells will be referred to as the MDA-MB-231 Oslo cell line.

Using the MDA-MB-231 Oslo cell line, the Oslo group plated the cells on glass coverslips and starved them in serum-free medium (SFM) for 4 hours. They then added a Src inhibitor to remove all invadopodia, followed by 4 hours of incubation in 10% FBS medium with HGF (50 ng/mL) to stimulate invadopodia re-formation (Pedersen et al., 2021).

In consideration of the promising results from the Oslo research group, we decided to perform degradation assays for both cell lines: the sample already available in the lab, along with the one received from the Oslo lab. To begin, each cell line was treated with either dimethyl sulfoxide (DMSO) as a positive control or 10uM of GM6001 (Galardin), a broad-spectrum metalloproteinase (MMP) inhibitor, to inhibit ECM degradation. It is important to remember that invadopodia degrades the extracellular matrix using matrix metalloproteinases (Jacob & Prekeris, 2015); thus, by inhibiting these molecules, degradation should be suppressed. The cells were plated in triplicate and incubated overnight for 16 hours as a preliminary screening for

degradation. According to this initial assay and general imaging, the original MDA-MB-231 cell line displayed more degradation than the Oslo line (Figure 1.3).

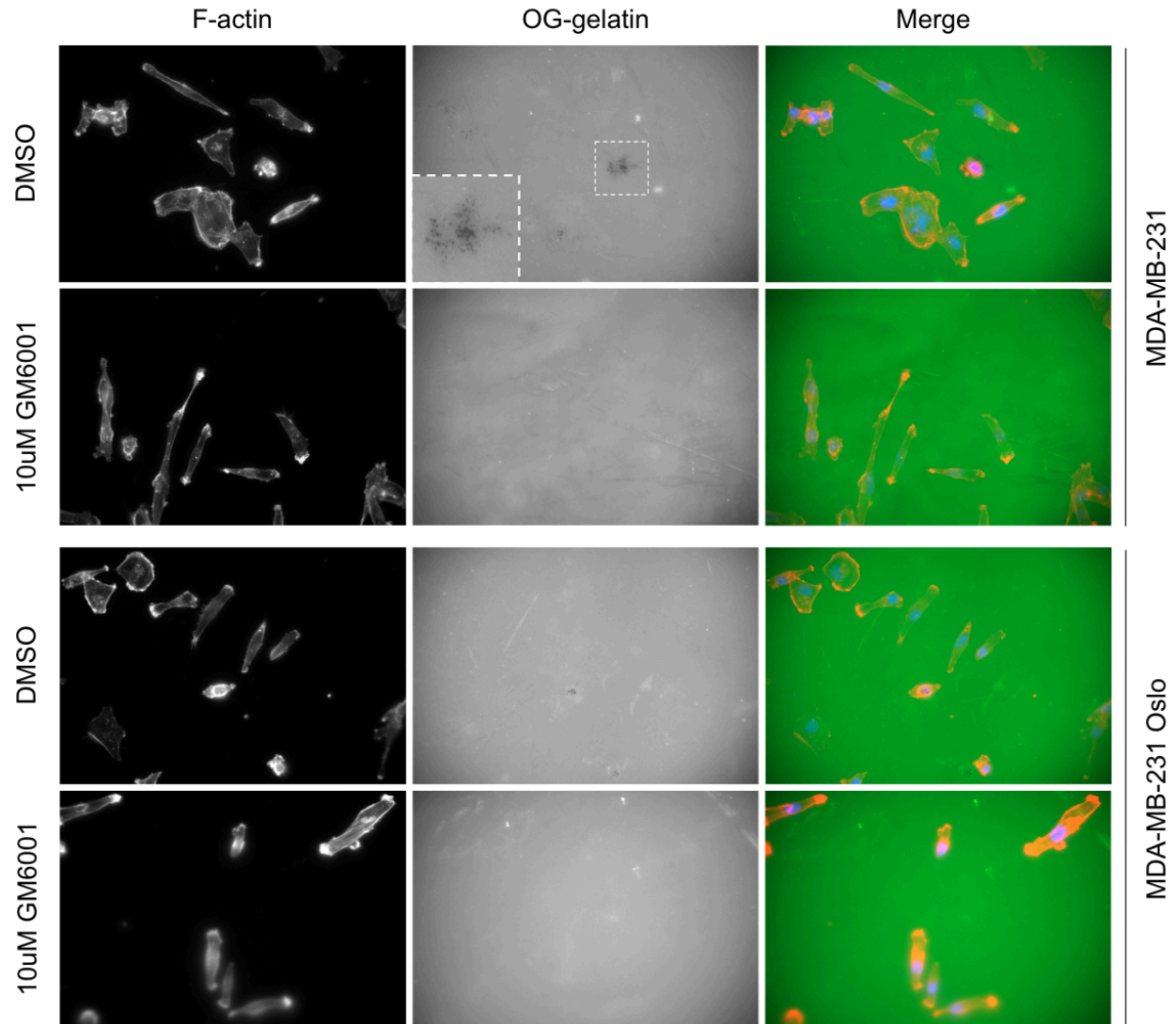


Figure 1.3: Matrix degradation assays for MDA-MB-231 and MDA-MB-231 Oslo cell lines.

2×10^4 cells/well of each cell line were plated and treated with either DMSO or 10uM GM6001.

Cells were then incubated on OG-gelatin coated coverslips for 16 hours. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. OG gelatin is shown in grayscale and areas of degradation appear as dark dots.

Note the degradation observed in the first DMSO-treated MDA-MB-231 cell line.

Next, we decided to repeat the experiment using the serum-free starvation and degradation re-stimulation process performed by the research group in Oslo in efforts to induce more degradation in both cell lines. Two samples of each cell line were treated again with DMSO and 10uM GM6001 as positive and negative controls, respectively, while additional samples were also treated with GM6001 to inhibit ECM degradation during the initial serum starvation phase. A schematic summary of this experimental design is shown below (Figure 1.4).

All samples were incubated overnight for 16 hours. Following incubation, the three samples of cells were starved in serum-free medium (SFM) for 4 hours; this starvation was used to synchronize the cells for epidermal growth factor (EGF) stimulation. One sample was immediately fixed with 4% paraformaldehyde (PFA). Both of the remaining samples were washed; one sample was incubated for an additional 4 hours in DMEM supplemented with 0.5% fetal bovine serum (FBS) as a means to monitor for invadopodia function with a minimal addition of growth factors, hormones, and nutrients, while the other sample was incubated for 4 hours in DMEM supplemented with 0.5% fetal bovine serum (FBS) and 2.5nM of EGF, which was reported to stimulate invadopodia formation (Figure 1.5).

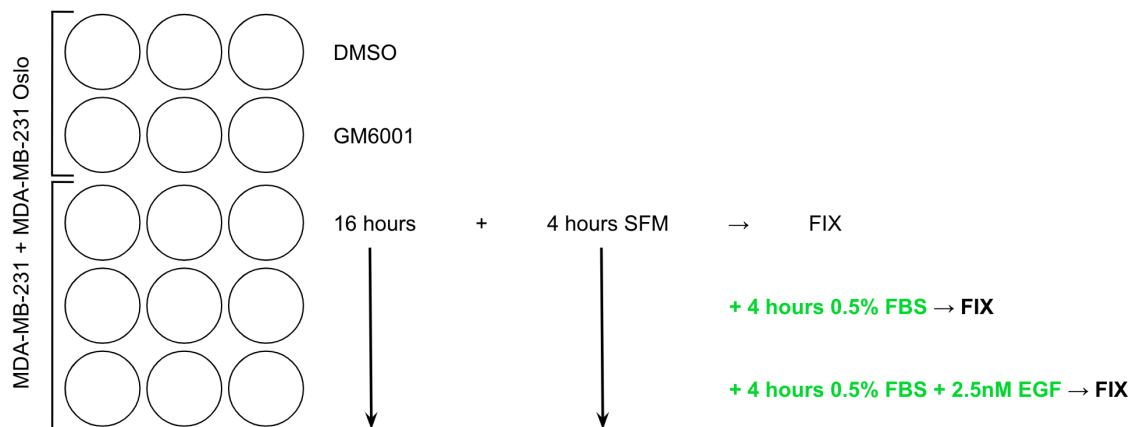
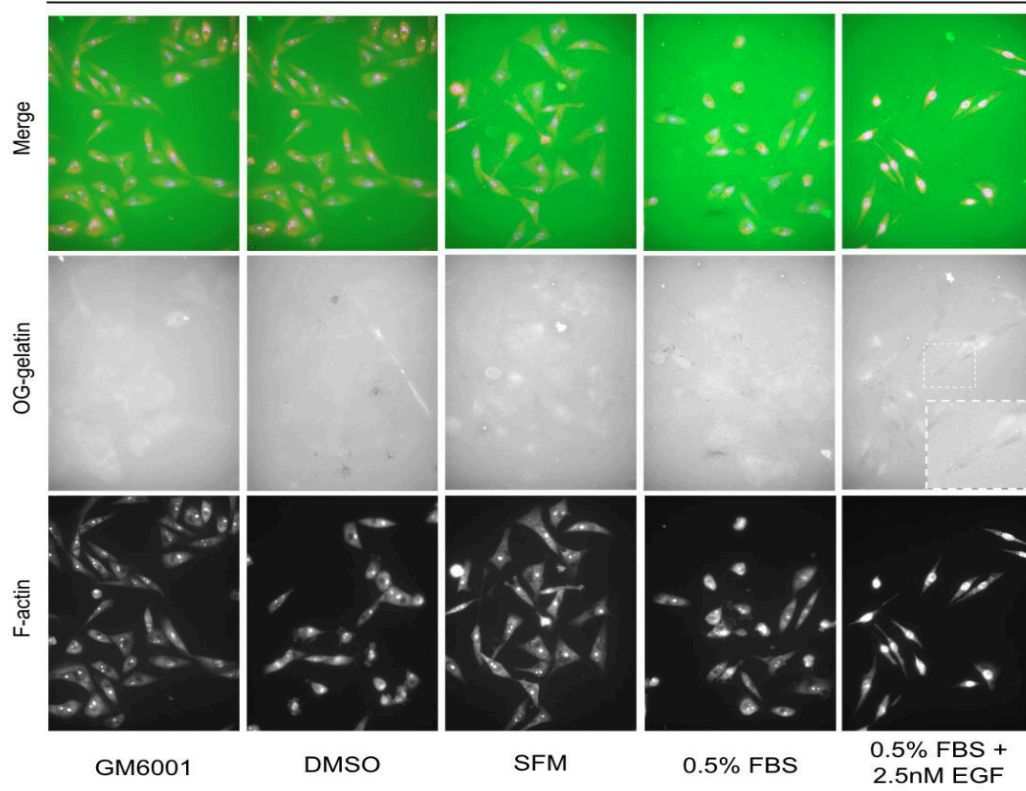


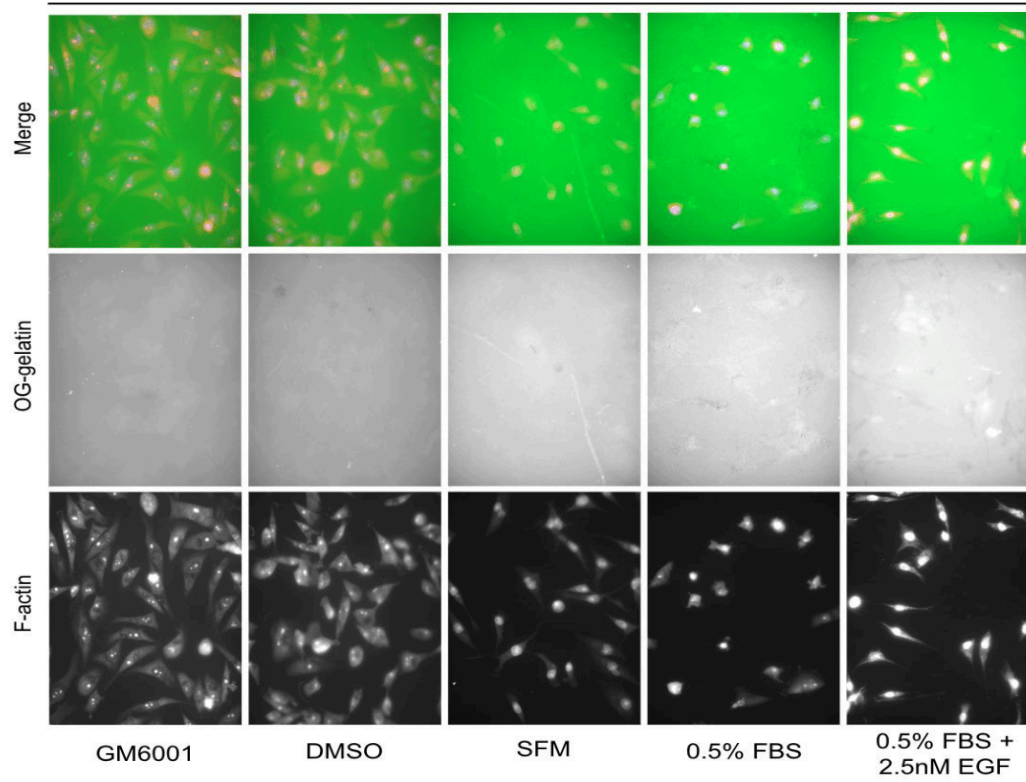
Figure 1.4: A schematic of the experimental protocol. Circles represent coverslips.

Figure 1.5: Matrix degradation assays for MDA-MB-231 and MDA-MB-231 Oslo cell lines with added treatments. 2×10^4 cells/well of each cell line were plated and treated with either DMSO or 10uM GM6001 as controls. All samples were then incubated on OG-gelatin coated coverslips for 16 hours. The two control samples were fixed following incubation, while the other three experimental samples were additionally incubated for 4 hours in SFM and 4 hours of 0.5% FBS or 0.5% FBS supplemented with 2.5nM EGF. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. OG gelatin is shown in grayscale and areas of degradation appear as dark dots.
Note the additional staining of filopodia in the OG-gelatin.

MDA-MB-231



MDA-MB-231 Oslo



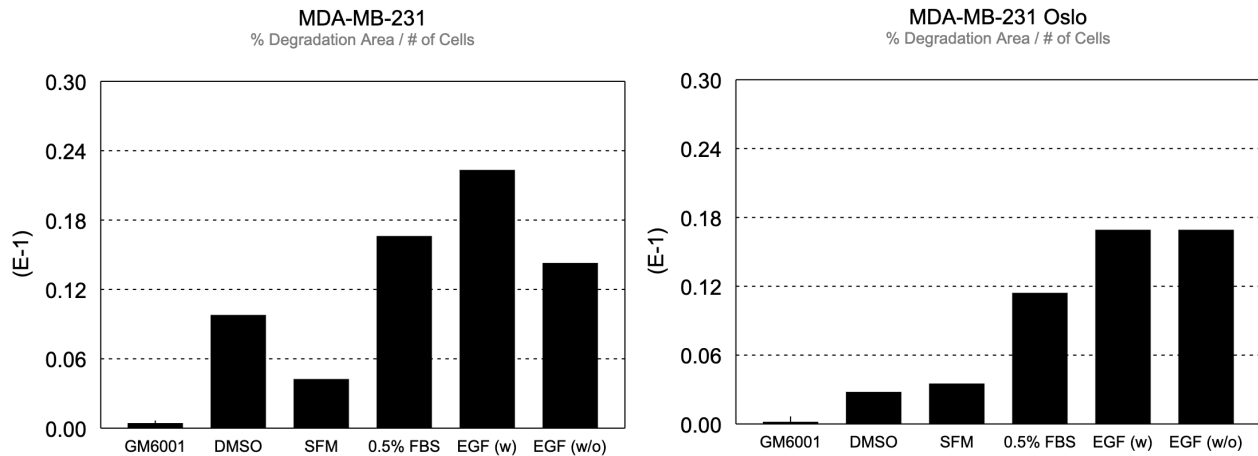


Figure 1.6: Quantification of matrix degradation assays for MDA-MB-231 cell lines. Total area of degradation was measured and normalized over the number of nuclei in each field to give the relative degradation values. Conditions tested include GM6001, DMSO, serum-free medium (SFM), 0.5% fetal bovine serum (FBS), and 0.5% FBS supplemented with EGF. Two methods were used to measure the additional staining of filopodia, labeled (w) for inclusion and (w/o) for exclusion. Note the y-axis scaled to $\times 10^{-1}$.

Quantification of the areas of degradation was performed with ImageJ (Figure 1.6). At least 30 fields were taken for each sample. The area of degradation per field was normalized by dividing the degradation area percentage by the total amount of cells in each field.

Unexpectedly, the degradation assay revealed additional potential degradation along the filaments of the cell cytoskeleton; these appeared as thin and shadow-like projections that followed the shape of the F-actin staining. We hypothesize that these distinct structures are filopodia, another protrusive structure very similar to invadopodia. They are both rich in actin, protrude from the cell surface, and play important roles in cell migration (Alblazi & Siar, 2015). Thus, the assumption can be made that the increased presence of EGF led to the potential formation of both structures due to their similar structural components.

To differentiate these two types of ECM degradation, I next performed two sets of quantification, including and excluding the shadows to be considered as degradation. In the

graph, bars marked as “EGF (w)” included the shadow-like filopodia in the area of degradation, while the bars marked “EGF (w/o)” did not include these structures.

My results demonstrated that EGF could potentially be used to induce invadopodia formation in both cell lines. However, since the MDA-MB-231 Oslo did not degrade as well in comparison to the original MDA-MB-231 cell line from the lab, we decided to no longer use it in future experiments.

Matrix degradation assays for MDA-MB-435 and MDA-MB-231 cell lines with additional treatments

Due to the promising degradation results from the EGF stimulation experiment, we decided to repeat the experiment with the original MDA-MB-231 cell line and the initial MDA-MB-435 cell line with additional condition changes.

Two samples of each cell line were treated again with DMSO and 25uM GM6001 as positive and negative controls, while additional samples were also treated with GM6001. A schematic summary of this experimental design is shown below (Figure 1.7). All samples were plated in triplicate and incubated overnight for 16 hours; control cells were then fixed with 4% PFA, while remaining sample cells continued with a 4 hour incubation in SFM followed by an additional 4 hour incubation in 0.5% FBS, 10% FBS (complete medium), 0.5% FBS supplemented with 2.5nM EGF, and 0.5% FBS supplemented with 10nM EGF.

Quantification of the controls was performed first to determine the overall degradation efficiency along with the inhibition efficiency from GM6001 (Figure 1.8). Both samples exhibited degradation with the DMSO treatment; while the MDA-MB-231 cell line had much more degradation overall in comparison to the MDA-MB-455 cell line (Figure 1.9).



Figure 1.7: A schematic of the experimental protocol. Circles represent coverslips. Changes in experimental conditions are highlighted in red.

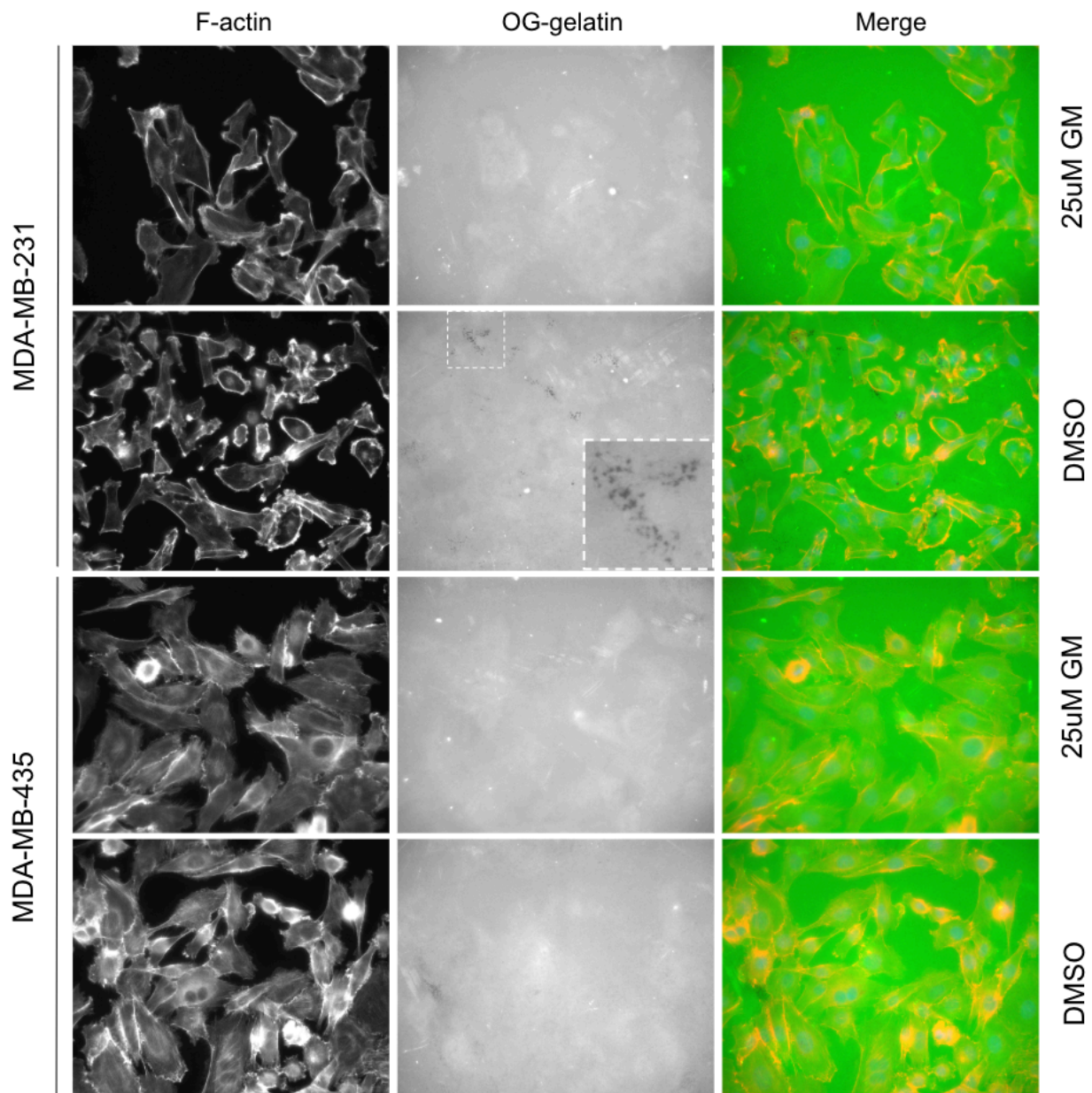


Figure 1.8: Matrix degradation assays for MDA-MB-231 and MDA-MB-435 controls. 2×10^4 cells/well of each cell line were plated and treated with either DMSO or 25uM GM6001 as controls. All samples were incubated on OG-gelatin coated coverslips for 16 hours. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. OG gelatin is shown in grayscale and areas of degradation appear as dark dots.

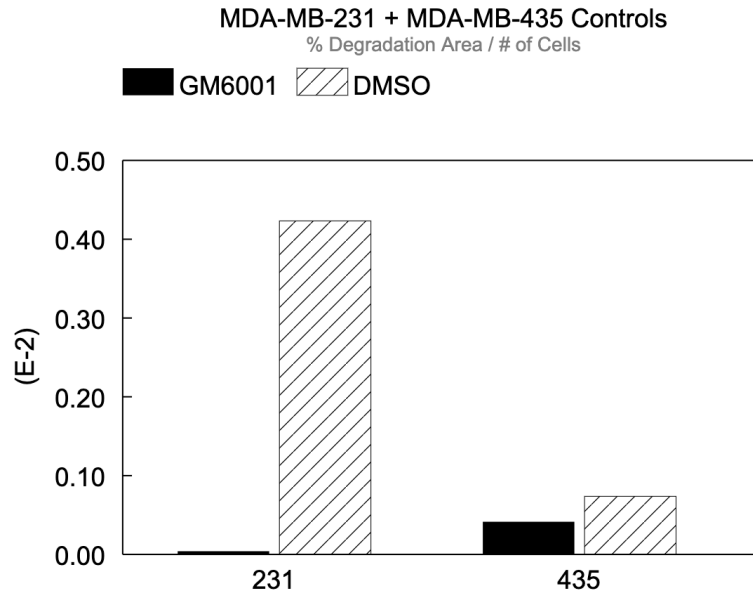
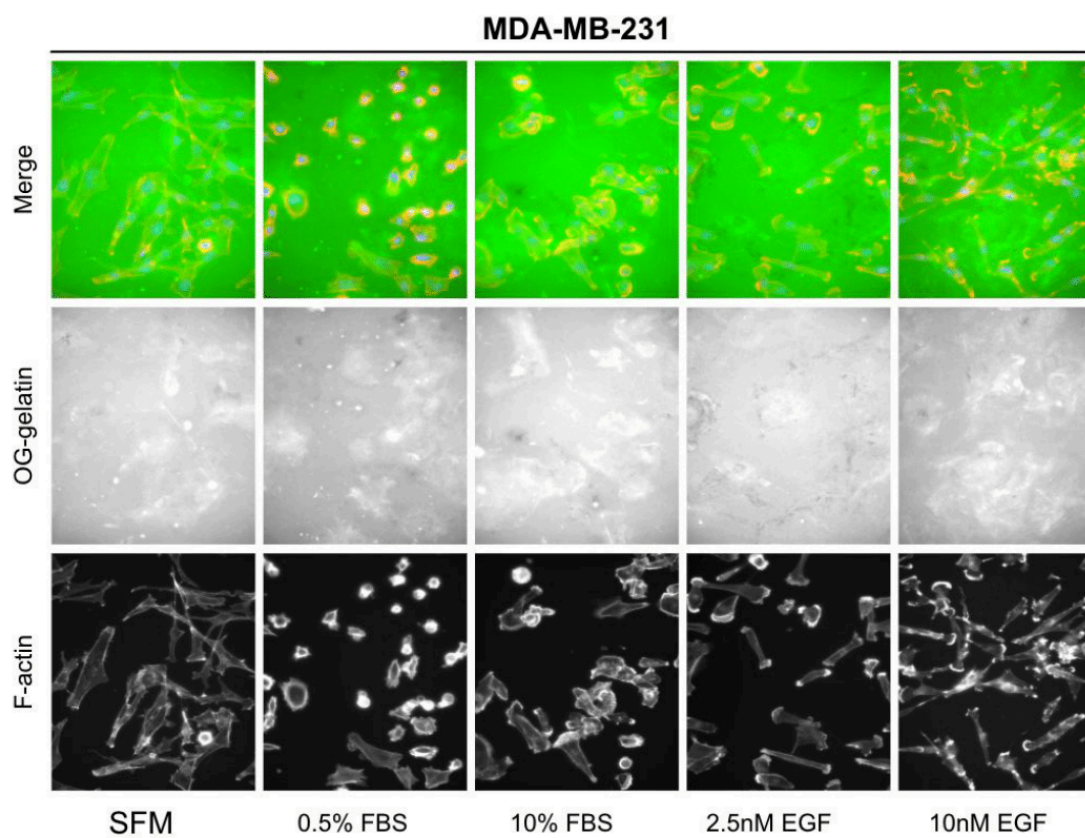
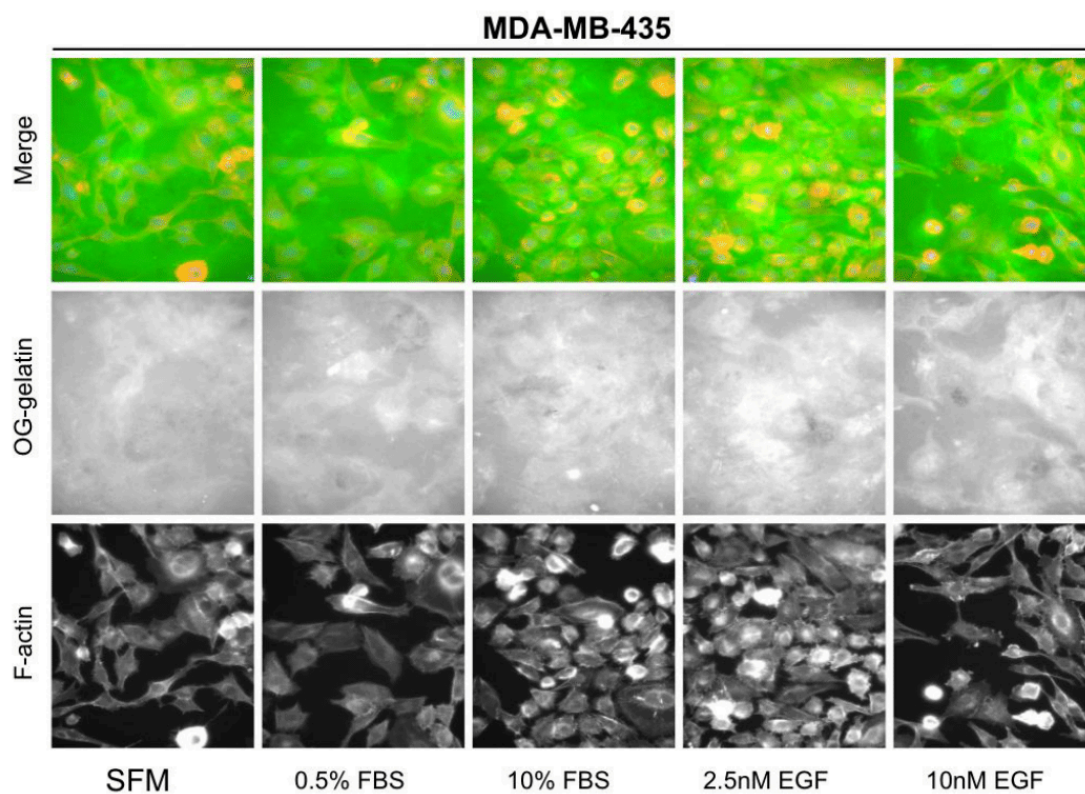


Figure 1.9: Quantification of matrix degradation assays for MDA-MB-231 and MDA-MB-435 cell line controls. Total area of degradation was measured and normalized over the number of nuclei in each field to give the relative degradation values. Note the y-axis scaled to $\times 10^{-2}$.

Additional images were taken of the other cell samples (Figure 1.10). At least 30 fields were taken for each sample, and areas of degradation were quantified using ImageJ (Figure 1.11). The area was normalized by dividing the degradation area percentage by the total amount of cells in each field. The relative area of degradation for the SFM control cells was set as 1 and the values for the other experimental cells were compared against it.

The MDA-MB-435 cell line demonstrated an increase in degradation after incubation in 0.5% FBS and 0.5% FBS supplemented with EGF. Between the two EGF concentrations, the 10nM EGF resulted in greater degradation, while the treatment with 0.5% FBS and 10nM resulted in similar degradation levels between the samples.

Figure 1.10: Matrix degradation assays for MDA-MB-231 and MDA-MB-435 cell lines with additional treatments. 2×10^4 cells/well of each cell line were plated and incubated on OG-gelatin coated coverslips for 16 hours. After, the experimental samples were additionally incubated for 4 hours in SFM and 4 hours of 0.5% FBS, 10% FBS, 0.5% FBS supplemented with 2.5nM EGF, or 0.5% FBS supplemented with 10nM EGF. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. OG gelatin is shown in grayscale and areas of degradation appear as dark dots.



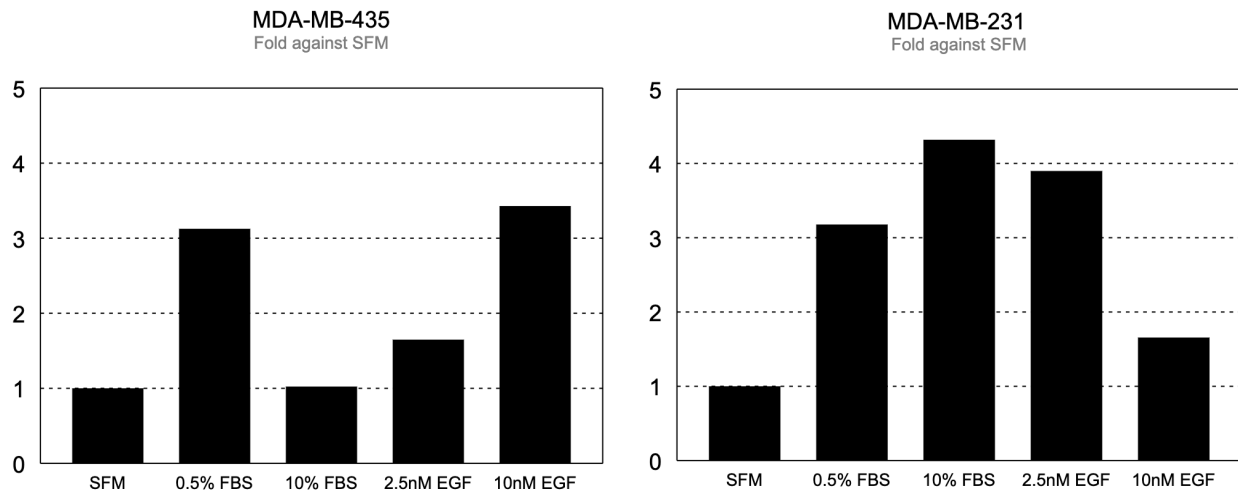


Figure 1.11: Quantification of matrix degradation assays for MDA-MB-231 and MDA-MB-435 cell lines with additional treatments. Total area of degradation was measured and normalized over the number of nuclei in each field to give the relative degradation values. Conditions tested include GM6001, DMSO, serum-free medium (SFM), 0.5% fetal bovine serum (FBS), and 0.5% FBS supplemented with EGF. Two methods were used to measure the additional staining of filopodia, labeled (w) for inclusion and (w/o) for exclusion. Note the y-axis scaled to $\times 10^{-1}$.

Meanwhile, the MDA-MB-231 cell line demonstrated an increase in degradation after all additive treatments when compared to the SFM control. Incubation in 10% FBS resulted in the greatest amount of degradation, followed by incubation in 2.5nM EGF. Unlike the other cell line, incubation in the 10nM EGF resulted in the least amount of degradation.

Due to the sufficient degradation rate in the samples solely incubated in FBS, we concluded that additional EGF stimulation may potentially not be required at all for degradation. The initial 4-hour serum-free starvation prior to supplementary incubation treatments also proved to be helpful in synchronizing the cells and encouraging invadopodia formation.

Additionally, based on the results of the degradation assay, the MDA-MB-435 cell line does not degrade well, particularly in comparison to the MDA-MB-231. This is the second time in which it has failed to degrade; therefore, I concluded that 435 cells are not suitable for ECM degradation studies.

Immunocytochemistry with MDA-MB-231 to detect Tks5

Due to stock issues, we were unable to replicate the previous gelatin degradation assay to verify the degradation capabilities of the MDA-MB-231 cell line. In particular, the Oregon Green (OG) 488 fluorescent dye previously utilized in the assay exhibited persistent precipitation issues that substantially impeded imaging quality; thus, it was deemed unusable for the time being, and we decided to utilize a different method in order to detect invadopodia formation in the cell line.

Immunocytochemistry is a technique used to detect specific proteins or antigens in cells with antibodies tagged with a fluorescent dye. In this way, the distribution and localization of target molecules are able to be visualized under fluorescent light (Renshaw, 2016). This experiment was performed by seeding the cells on gelatin-coated coverslips, treating the cells with a permeabilizing agent, and blocking with a bovine serum albumin (BSA) solution before lastly incubating with a primary and secondary antibody.

Tks5 is a tyrosine kinase substrate with multiple Src homology domains. Tks5 adaptor proteins are crucial as scaffold proteins for invadopodia, and Tks5 localizes to invadopodia in human cancer cells. It plays an integral role in invadopodia formation, making it an ideal candidate to detect in order to find invadopodia-mediated degradation (Diaz, et al., 2009).

To start, we seeded the MDA-MB-231 cells on porcine gelatin-coated coverslips with a concentration of 2×10^4 cells/well. DMSO and GM6001 were used as positive and negative controls, respectively. All samples were incubated overnight for 16 hours, and experimental samples were starved in serum-free medium (SFM) for 4 hours, followed by hourly increments of incubation in complete medium (10% FBS), from 1-4 hours. This was done in order to locate the co-localization of invadopodia with the cell structure prior to the cells moving away, since

the MDA-MB-231 cells were highly motile. After the treatments, samples were fixed and permeabilized before being incubated in a Tks5 primary antibody, then a HRP secondary antibody. Additional staining was performed with phalloidin for F-actin and DAPI for the cell nucleus. Lastly, the samples were mounted and imaged using a fluorescence microscope (Figure 1.12).

The images taken of the samples were all very dark, even when taken with a long exposure time. Tks5, the protein of interest, should appear in the Tks5 fields as white dots or areas, yet the fields are broadly dim. Thus, we concluded that this could be attributed to human error during plating, or that the MDA-MB-231 cell line as a whole does not express Tks5 during invadopodia-mediated degradation.

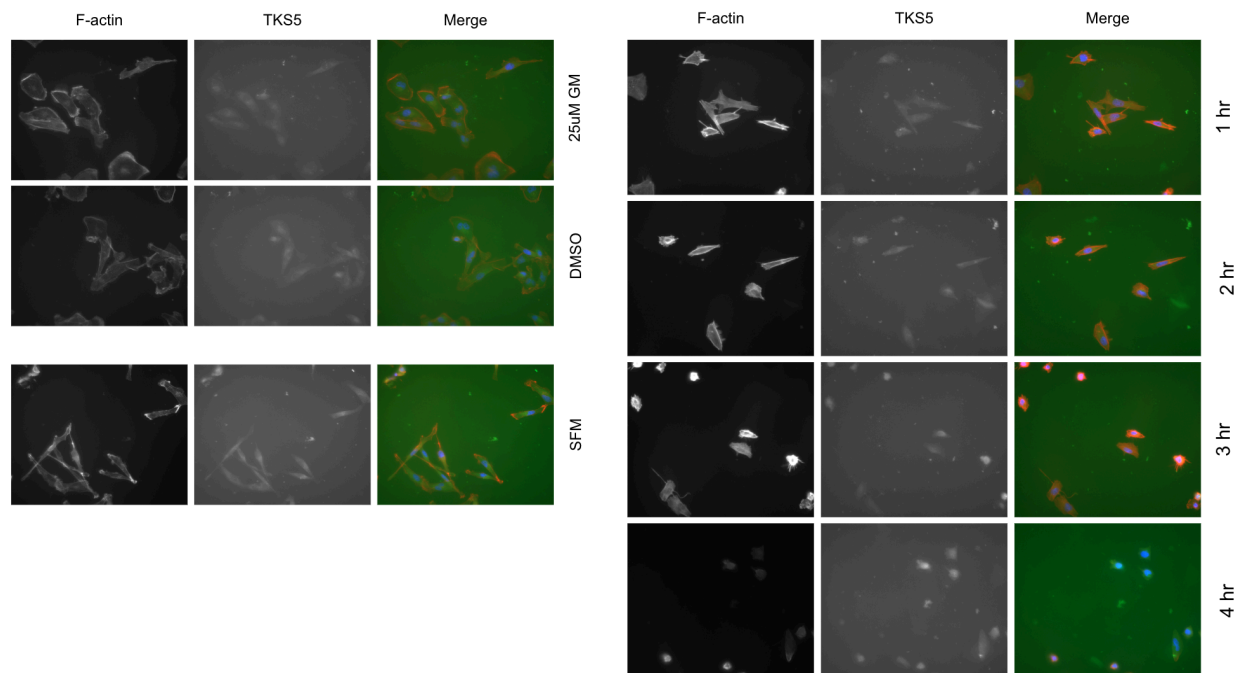


Figure 1.12: Immunocytochemistry assay for MDA-MB-231. Cells were plated with 2×10^4 cells/well and incubated for 16 hours on porcine-gelatin coated coverslips. DMSO, GM6001, and SFM were used as controls. The remaining samples were incubated in complete media for 1-4 hours. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. Note the lack of brightly colored spots in the Tks5 fields.

Western blot analysis for Tks5 and Cortactin

To evaluate whether the MDA-MB-231 cell line expressed Tks5 and to quantify its expression, given the poor fluorescence observed in the immunocytochemistry assay, we decided to perform a Western blot. Western blots are used to detect and quantify specific proteins in a sample; in this case, we specifically probed for Tks5. Alongside the MDA-MB-231 cell line, we also included the initial MDA-MB-435 cell line and SCC61 cell line to serve as an additional experimental sample for comparison (Figure 1.13a).

To start, we extracted protein from all cell lines and quantified them using a Lowry assay. The protein samples were then loaded onto an SDS-PAGE gel where they were separated and then transferred onto a PVDF membrane. 20ug, 40ug, and 80ug of each protein sample was loaded onto the gel, with the exception of the SCC61 cell line, for which only 20ug was loaded since it served as the control and was already known to effectively form invadopodia. The membrane was then blocked in a BSA solution, incubated in a Tks5 primary antibody, and incubated in HRP secondary antibody. After probing for the protein of interest, the membrane was incubated again in primary and secondary antibodies specific to GAPDH, a loading control used to ensure equal protein loading and transfer across samples. For the last step, protein signals were detected using chemiluminescence.

ImageJ was used to measure the intensity of each protein band and to quantify them. The intensity of the target protein bands were divided by the intensity of the GAPDH bands in order to normalize them (Figure 1.13b). The SCC61 control exhibited nearly three times higher expression of Tks5 compared to the other samples, confirming its extensive invadopodia formation. The normalized Tks5 expression levels demonstrate consistency across different protein loadings for both cell lines, with slightly increased expression observed at higher protein concentrations; however, the overall expression was still significantly low compared to the SCC61 control. Given these outcomes, we concluded that Tks5 was indeed poorly expressed in both the MDA-MB-231 and MDA-MB-435 cell lines.

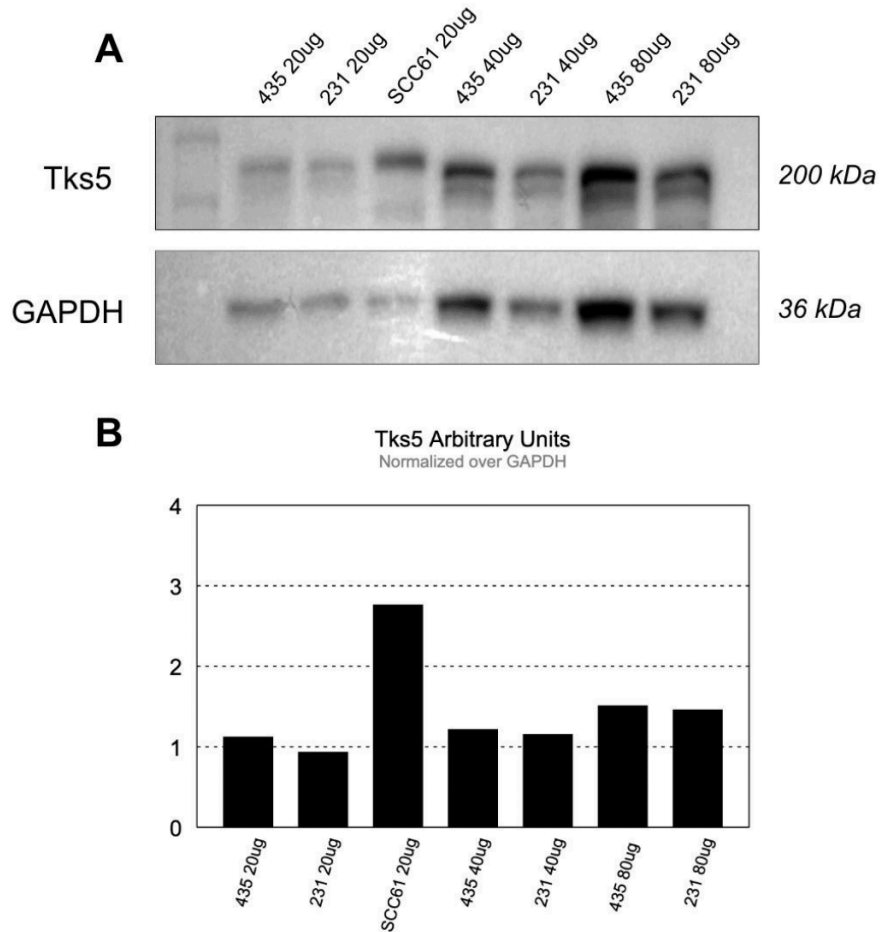


Figure 1.13: Western blot analysis and quantification for Tks5. (A) Western blot showing the expression levels of Tks5 and GAPDH across different cell lines and protein loading concentrations. The cell lines used are MDA-MB-435, MDA-MB-231, and SCC61. GAPDH was used as a loading control. (B) Quantification of Tks5 expression normalized to GAPDH using arbitrary units.

Following the unsuccessful probing for Tks5, we decided to probe for Cortactin instead. Cortactin is a large adaptor protein that promotes actin polymerization, a critical process for forming protrusive structures such as invadopodia. Given its involvement in every step of invadopodia formation, Cortactin is an ideal protein to detect for correlating with invadopodia formation (Jeannot & Besson, 2017).

The same PVDF membrane as the previous experiment was used in order to assess for Cortactin. The membrane was incubated in a Cortactin primary antibody followed by a HRP

secondary antibody before being imaged with chemiluminescence (Figure 1.14a). ImageJ was used again to measure the intensity of each protein band, and they were normalized over GAPDH in order to be quantified (Figure 1.14b).

Unlike the previous Western blot results, the SCC61 control exhibited relatively similar amounts of Cortactin expression compared to the other samples. All of the samples generally ranged between the arbitrary values of 1.5-2. Based on these results, we concluded that Cortactin was much more consistently expressed across all cell line samples, especially when in comparison to Tks5.

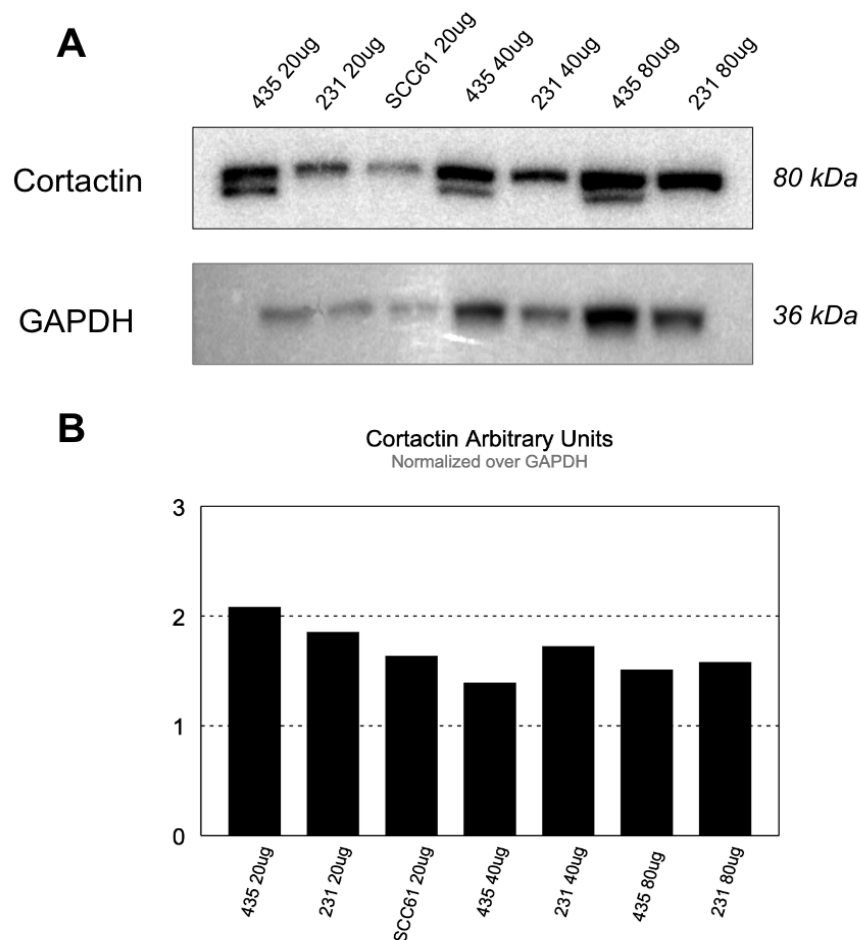


Figure 1.14: Western blot analysis and quantification for Cortactin. (A) Western blot showing the expression levels of Cortactin and GAPDH across different cell lines and protein loading concentrations. The cell lines used are MDA-MB-435, MDA-MB-231, and SCC61. GAPDH was used as a loading control. (B) Quantification of Cortactin expression normalized to GAPDH using arbitrary units.

Final matrix degradation assay for MDA-MB-231 cell line with complete media incubation

Considering the results from both of the Western blots, we ultimately decided to perform one final matrix degradation assay using the MDA-MB-231 as a final attempt to induce invadopodia formation. Since the incubations in complete medium from the immunocytochemistry assay performed well, we repeated the same experimental setup.

MDA-MB-231 cells on porcine gelatin-coated coverslips were seeded with a

concentration of 2×10^4 cells/well. DMSO and GM6001 were used as positive and negative controls, respectively. All samples were plated in triplicate and incubated overnight for 16 hours; experimental samples were starved in serum-free medium (SFM) for 4 hours, followed by hourly increments of incubation in complete medium (10% FBS), from 1-4 hours. A schematic summary of this experimental design is shown below (Figure 1.15).

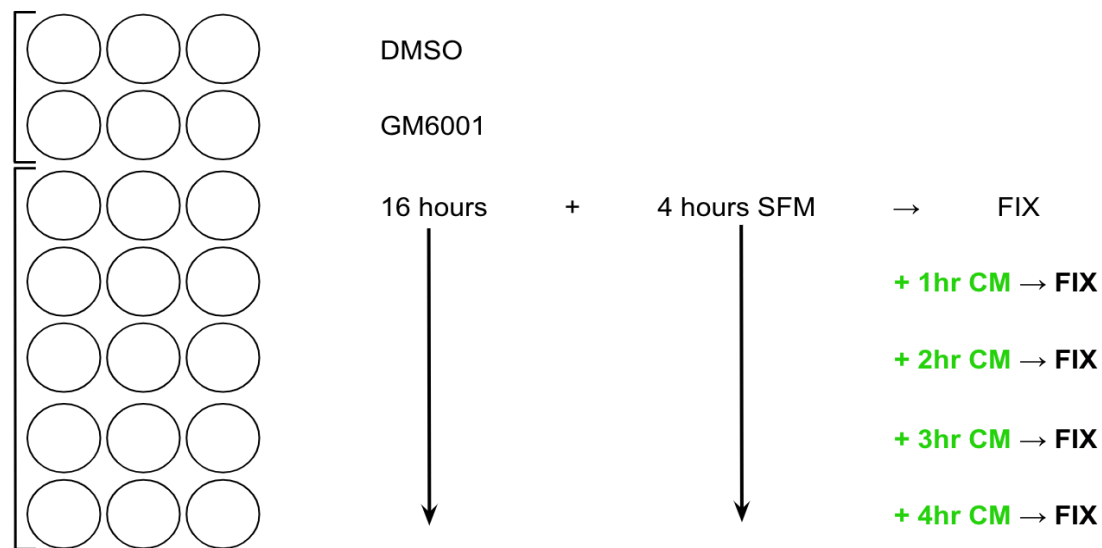


Figure 1.15: A schematic of the experimental protocol. Circles represent coverslips. “CM” represents complete medium.

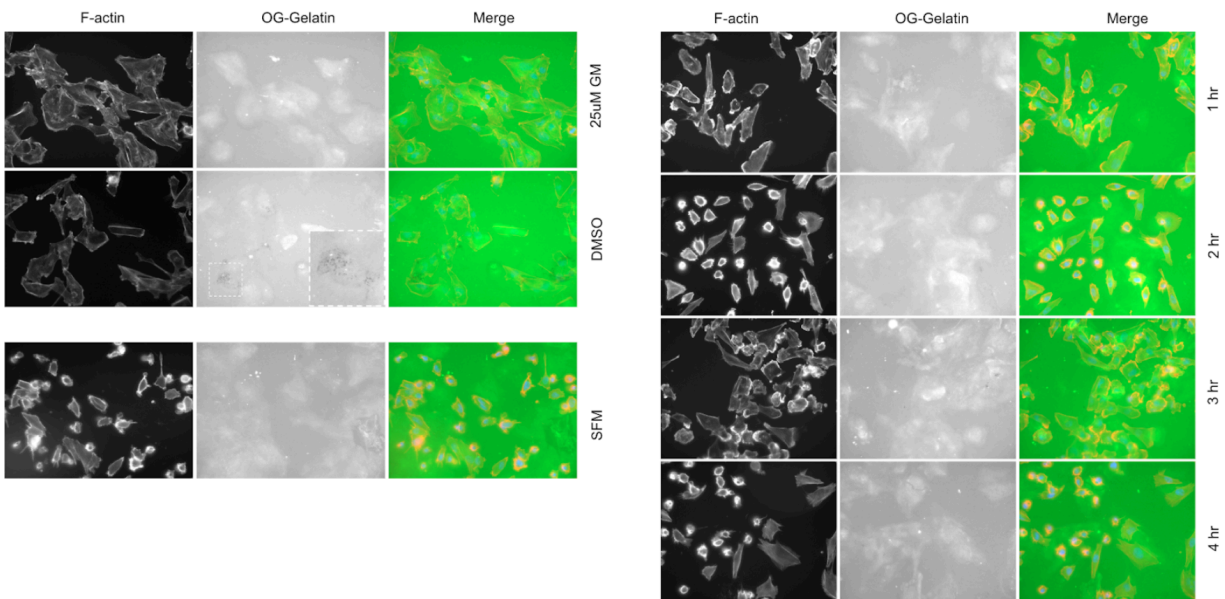
The cell samples were imaged using fluorescence and quantified using ImageJ (Figure 1.16). Again, the area was normalized by dividing the degradation area percentage by the total amount of cells in each field.

The results demonstrated that the controls responded as expected, with GM6001 effectively inhibiting degradation while DMSO allowing ample degradation. Contrary to our expectations, however, the samples treated with DMSO essentially degraded even better than the other samples incubated in complete medium for varying time points following serum-free

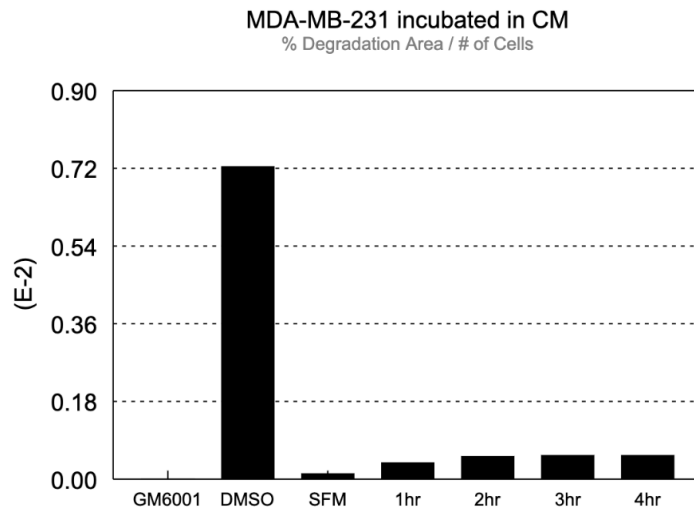
starvation. Due to these results after the various experimental conditions we utilized in order to induce degradation, we ultimately concluded that the degradation capacity of the MDA-MB-231 cell line is not as high as desired. Thus, the decision was made to move on and switch to new and different cell lines.

Figure 1.16: Matrix degradation assay and quantification for MDA-MB-231 cell line with complete medium incubation. (A) Cells were plated with 2×10^4 cells/well and incubated for 16 hours on OG-gelatin coated coverslips. DMSO, GM6001, and SFM were used as controls. The remaining samples were incubated in complete media for 1-4 hours. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. (B) Total area of degradation was measured and normalized over the number of nuclei in each field to give the relative degradation values. Note the y-axis scaled to $\times 10^{-2}$.

A



B



Invadopodia are small, actin-rich protrusions that penetrate into the extracellular matrix. Their ability to degrade the ECM makes them a crucial player in the epithelial-to-mesenchymal transition (EMT), which contributes to its ability to facilitate cancer metastasis. However, its presence in various cancer cell lines has not been widely researched. This preliminary study focused on the MDA-MB-435 (melanoma) and MDA-MB-231 (highly aggressive triple-negative breast cancer) cell lines, testing them under various conditions to investigate their ECM degradation abilities.

The MDA-MB-435 cell line was assessed first using various matrix degradation assays. The first assay showed little to no degradation; in order to confirm these findings, further experiments were repeated with additional conditions (such as longer incubation time, adding EGF, serum starvation) as an attempt to bolster ECM degradation potential. However, the cell line continued to show poor degradation performance under all conditions tested, even though the Western blot exhibited the presence of Cortactin, a key regulator in invadopodia formation. The consistently poor degradation results for the MDA-MB-435 cell line, especially when compared to the SCC61 positive control cell line, led to the conclusion that this cell line does not degrade the ECM effectively. Consequently, the decision was made to switch to another cell line for further studies.

The MDA-MB-231 and MDA-MB-231 Oslo cell lines were studied next. It must be remembered that these two are inherently the same cell line; however, the “Oslo” line came from a research group in Oslo, Norway that was able to effectively observe degradation in their experiments through serum-free starvation and degradation re-stimulation. Both cell lines were

used simultaneously for matrix degradation assays, and initial assays show sufficient ECM degradation, particularly when treated with DMSO as a positive control. Using the experimental structure of the Oslo research group, a similar treatment model was emulated and conducted on both cell lines; treatments included serum-free starvation, additional incubation in a medium supplemented with EGF at varying concentrations, and more. Surprisingly, the cell line from the Oslo group exhibited much less degradation than the original cell line from the lab; thus, the decision was to only move forward with using the original cell line. Additional Western blot analysis indicated that the cell line also exhibited the presence of Cortactin; however, following a final degradation assay to validate our findings, the results indicated that the degradation was less substantial than anticipated. Consequently, we decided to proceed with testing other cell lines.

Furthermore, even though the Western blot analysis revealed that Cortactin showed relatively consistent expression across all tested cell lines, it also revealed that Tks5 was poorly expressed in both cell lines of interest, especially when compared to the SCC61 positive control cell. ITCH, an E3 ubiquitin ligase, plays a crucial role in regulating proteins involved in invadopodia formation. These findings thus suggest that ITCH may have the potential to influence invadopodia formation indirectly through its regulation of proteins similar to Tks5 and Cortactin. Given the low expression of Tks5 in both cell lines, ITCH's role could be more significant in other cell lines where Tks5 is expressed at higher levels. Additionally, due to the similar Cortactin levels across all cell lines, Cortactin itself might not be a limiting factor in invadopodia formation in these cell lines. Overall, the differential expression of Tks5 and consistent expression of Cortactin highlight ITCH's potential selective influence on invadopodia-associated proteins. Also, ITCH's potential regulation of invadopodia formation

through these proteins opens the possibility of its broader impact on overall cancer cell invasion and metastasis through the modulation of key proteins involved in invadopodia formation.

Throughout the study, there were several obstacles that arose. The primary issue encountered with the Oregon green (OG) 488 dye was its persistent precipitation problems, which significantly impaired the imaging quality. This issue rendered the dye unusable for the degradation assays at the time, creating a substantial obstacle for visualizing and quantifying the assays. This necessitated a change in methodology to ensure more accurate results; therefore, immunocytochemistry was chosen as an alternative method to detect specific proteins associated with invadopodia. Western blot was also additionally used to quantify relevant protein expression; this method allowed for more reliable means of assessing protein levels involved in invadopodia formation and function. Another issue that arose was the lack of response from the cell lines following various treatments to induce invadopodia formation. The initial treatments, which involved additional incubation in 0.5% FBS and 0.5% FBS supplemented with 2.5nM EGF, resulted in insubstantial results, resulting in the decision to add more variables. On top of the previous treatments, additional samples were incubated in 10% FBS as well as 0.5% FBS supplemented with 10nM EGF as a means to determine if increased EGF will facilitate greater degradation amounts. Overall, these methodological adjustments were crucial in addressing and overcoming the challenges encountered. These changes not only aimed to enhance the accuracy of degradation measurements but also ensured the reproducibility of results, thereby contributing to a more robust and reliable assessment of invadopodia-mediated ECM degradation in these specific cell lines.

While the results provided valuable insights, several limitations must be considered. For one, the variability of the degradation assays, particularly the inconsistent degradation results

observed in different cell lines, highlighted the potential issues with reproducibility and experimental consistency. Human error in plating and imaging could have also played a significant role in the inconsistencies found in the results. Also, the ability of the different cell lines to degrade could either be contributed to these errors or they could be due to intrinsic differences between the cell lines' ability to degrade at all. In order to mitigate these limitations in future studies, experimental protocols should be standardized and strictly followed to reduce variability. Conducting the same experiments with larger sample sizes and more replicates can also be done in order to ensure statistically significant and reproducible results.

Based on the results of this preliminary screening, we decided to extend our research to encompass additional and more aggressive cancer cell lines.

CHAPTER 2: ADDITIONAL CELL LINE SCREENING & ITCH KNOCKDOWN ANALYSIS TO INVESTIGATE THE FUNCTION OF ITCH IN METASTASIS

This second chapter will examine the invadopodia-mediated degradation abilities of four additional cell lines: A673 (Ewing sarcoma), EWS-502 (Ewing sarcoma), TC-32 (Ewing sarcoma), and SK-N-MC (Ewing sarcoma). Furthermore, it will discuss whether ITCH knockdown impacts invadopodia formation and ECM degradation.

The A673 cell line is a highly aggressive Ewing sarcoma cell line; Ewing sarcoma is a rare and very invasive form of bone cancer primarily affecting children and young adults. It has been well-studied and grows robustly in cell culture (Teicher et al., 2011). Meanwhile, the EWS-502 cell line is another highly metastatic, patient-derived Ewing sarcoma cell line (Sankar et al., 2014). TC-32 is a lesser-studied primitive neuroectodermal tumor (PNET) cell line that is also used as a model for Ewing sarcoma (Pagani et al., 1992), and SK-N-MC is another cell line derived from a human Ewing sarcoma tumor (Abadie et al., 1985). The literature indicates that the A673 cell line exhibits high levels of invadopodia-mediated ECM degradation (Bailey et al., 2016). Furthermore, previous studies from our lab have demonstrated that this cell line, along with the others, possesses highly invasive characteristics. These findings suggest their significant potential for invadopodia formation and ECM degradation studies.

Upon confirming which cell lines exhibit sufficient levels of degradation, we will conduct an ITCH knockdown to elucidate its role in invadopodia formation and its contribution to ECM degradation within each respective cell line.

Matrix degradation assays for 4 cell lines

To initially assess the degradation abilities of the four cell lines (A673, EWS-502, TC-32, and SK-N-MC), two concentrations of each cell line were plated in triplicate overnight for 16 hours on OG-gelatin coated coverslips. The same 2D-fluorescent matrix degradation assay protocol was performed, as in Chapter 1. Following this overnight incubation, the coverslips were processed and imaged. The resulting images from all cell lines indicated minimal to no degradation.

After this, the same experiment was repeated with increased incubation times. Cells were plated in triplicate with the concentration of 4×10^4 cells/well for 16 hours, 4×10^4 cells/well for 24 hours, and 2×10^4 for 48 hours. The EWS-502 and SK-N-MC cell lines were incubated for 16 hours again due to their slight degradation observed in the initial preliminary assay. In contrast, the other cell lines were incubated for longer periods immediately, as they showed no degradation in the first assay. Furthermore, fewer cells were plated for the extended 48-hour incubation period to account for cell growth.

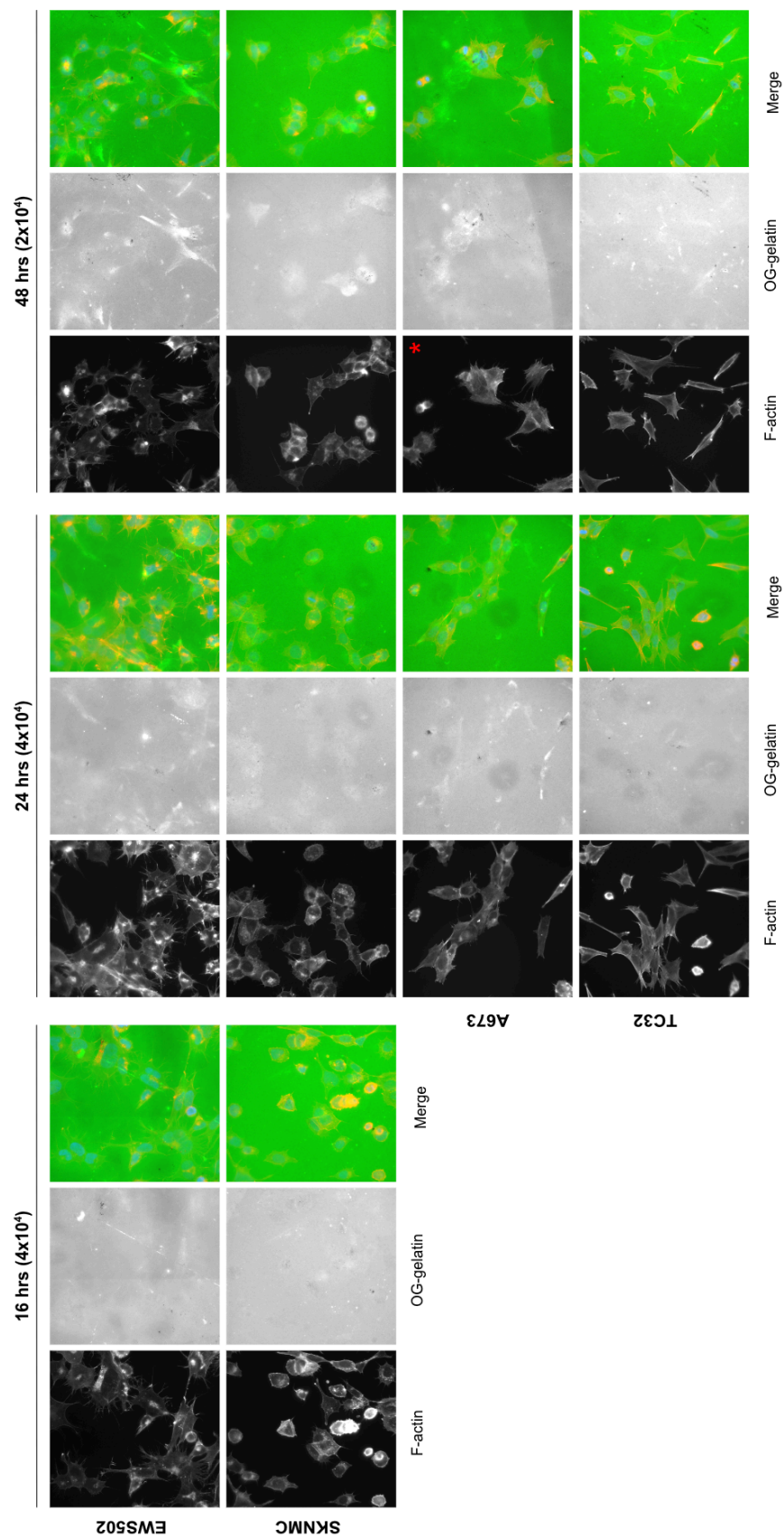
The coverslips were subsequently processed and imaged as previously described (Figure 2.1). Additionally, they were quantified using ImageJ to gain a more comprehensive understanding of their degradation potential (Figure 2.2). As before, at least 30 fields were captured for each sample. The degradation area per field was normalized by dividing the degradation area percentage by the total number of cells in each field. Notably, only the A673 cell line sample from the 48-hour incubation was quantified. The other cell lines, being highly mobile, were omitted from quantification as their mobility typically leads to inaccurate degradation measurements.

The results demonstrated that at the 16-hour degradation time, minimal degradation was observed in the EWS-502 cell line, while the SK-N-MC cell line exhibited slight degradation, consistent with preliminary findings. After 24 hours, the A673 cell line demonstrated moderate degradation. The EWS-502 cell line showed increased degradation compared to its 16-hour incubation results, and the SK-N-MC cell line exhibited a very slight increase in degradation compared to its 16-hour incubation. The TC-32 cell line showed minimal degradation after 24 hours. Notably, the A673 cell line displayed significant degradation after 48 hours, highlighting its strong degradation potential over extended incubation periods and its overall strong invadopodia formation potential. The other cell lines demonstrated minimal to moderate degradation, but not as strong as the A673 cell line.

After analyzing the quantified data and visually inspecting the images of each cell line, we determined that the A673 and EWS-502 cell lines exhibited the highest degradation abilities. Consequently, we decided to use these cell lines for our ITCH knockdown experiment.

Figure 2.1: Matrix degradation assays for the A673, EWS-502, TC-32, and SK-N-MC cell lines. Cells were plated on OG-gelatin coated coverslips with the concentration of 4×10^4 cells/well for 16 hours, 4×10^4 cells/well for 24 hours, and 2×10^4 cells/well for 48 hours. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. OG-gelatin is shown in grayscale and areas of degradation appear as dark dots.

*Only cell line quantified for the 48-hour incubated samples.



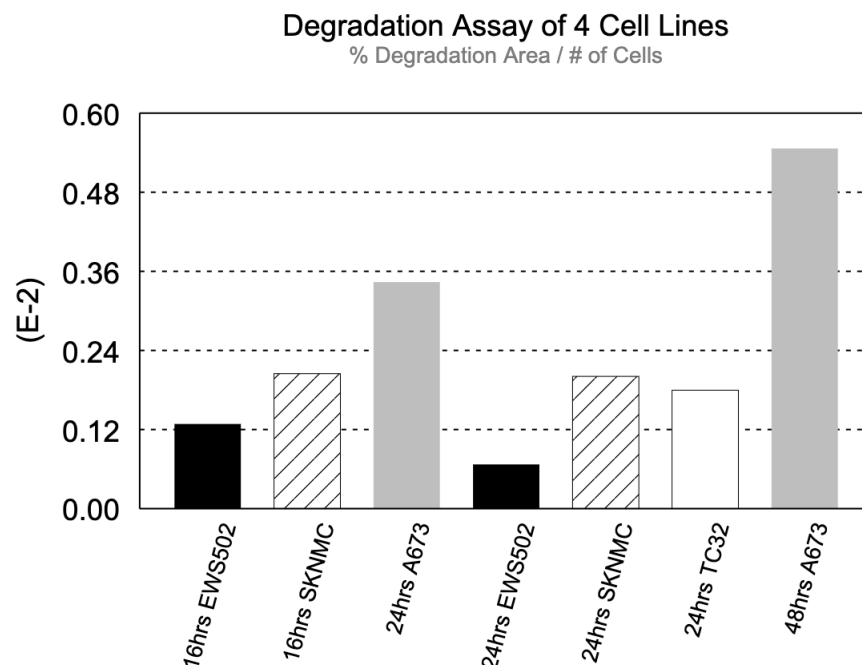


Figure 2.2: Quantification of matrix degradation assays for the A673, EWS-502, TC-32, and SK-N-MC cell lines with various incubation times. Total area of degradation was measured and normalized over the number of nuclei in each field to give the relative degradation values. Three different time points were tested: 16 hours, 24 hours, and 48 hours. Only the A673 cell line sample was quantified for the 48-hour incubation. Note the y-axis scaled to $\times 10^{-2}$.

ITCH Knockdown & Degradation Assay Analysis

To investigate the role of the ITCH protein in their invadopodia-forming abilities, we decided to perform an ITCH protein knockdown using small hairpin RNA (shRNA) transfection. By knocking down ITCH, we aim to observe changes in degradation levels, thereby gaining insight into the role ITCH plays in invadopodia function.

We infected A673 and EWS-502 cells with various constructs, including shSCR (scrambled shRNA), shRFP (control shRNA targeting red fluorescent protein), GFP (control lentiviral vector expressing green fluorescent protein), and three different shITCH constructs (shITCH #1, #2, and #6). shSCR and shRFP were used as positive controls, and shGFP was used to monitor infection efficiency. These cells were also infected with different viral concentrations

of 1:2, 1:8, and 1:30. Following the infection, the knockdown of ITCH levels was confirmed via quantitative PCR (qPCR) (Figure 2.3). shSCR was used as the standard and was set to 1; knockdown efficiency was categorized as being less than 0.5. Additionally, shGFP does not have the ability to uptake or incorporate viral plasmid DNA and was only used as a monitoring tool, so it was not quantified for knockdown efficiency.

Unfortunately, multiple infected cell samples were rendered unusable due to poor growth or contamination. For the remainder of the experiment, only the cell samples that were healthy enough for RNA extraction and qPCR were utilized.

The results from qPCR demonstrated that the ITCH knockdown for the 1:8 dilution of the A673 cell line was quite effective, with the cells transfected with the shITCH #2 construct being much lower compared to the shSCR control construct and the threshold considered to be an efficiency knockdown (0.5). Meanwhile, the EWS-502 cell line had two surviving dilutions. The 1:8 dilution contained healthy constructs for all shRNAs, while the 1:30 dilution lacked healthy cells infected with the shRFP and shITCH #2 constructs; thus, they were not quantified. Only the shITCH #2 construct in the 1:8 dilution exhibited sufficient knockdown levels, defined as less than 0.5.

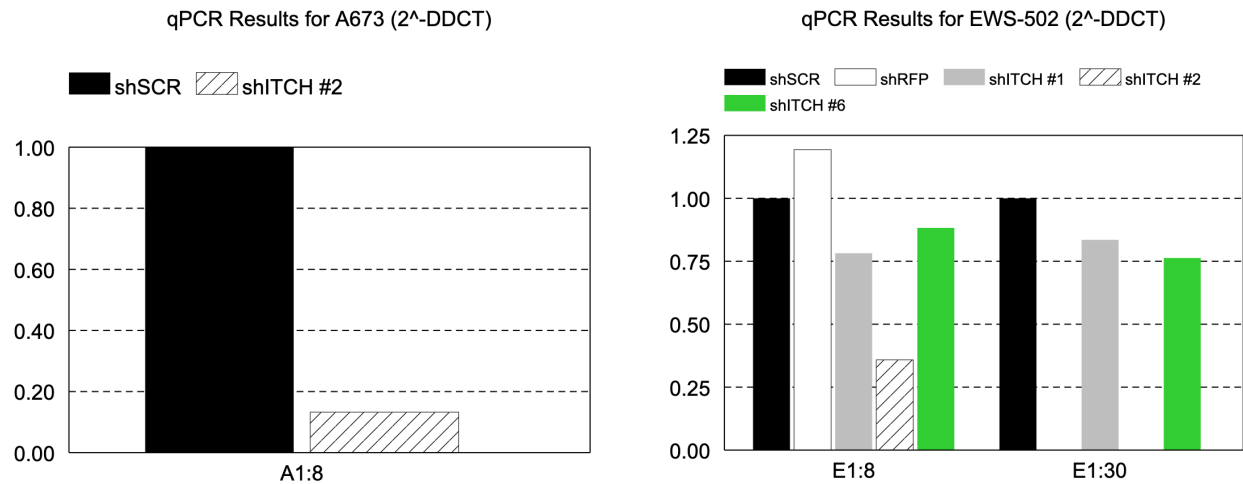


Figure 2.3: ITCH knockdown efficiency for the A673 and EWS-502 cell line through qPCR.

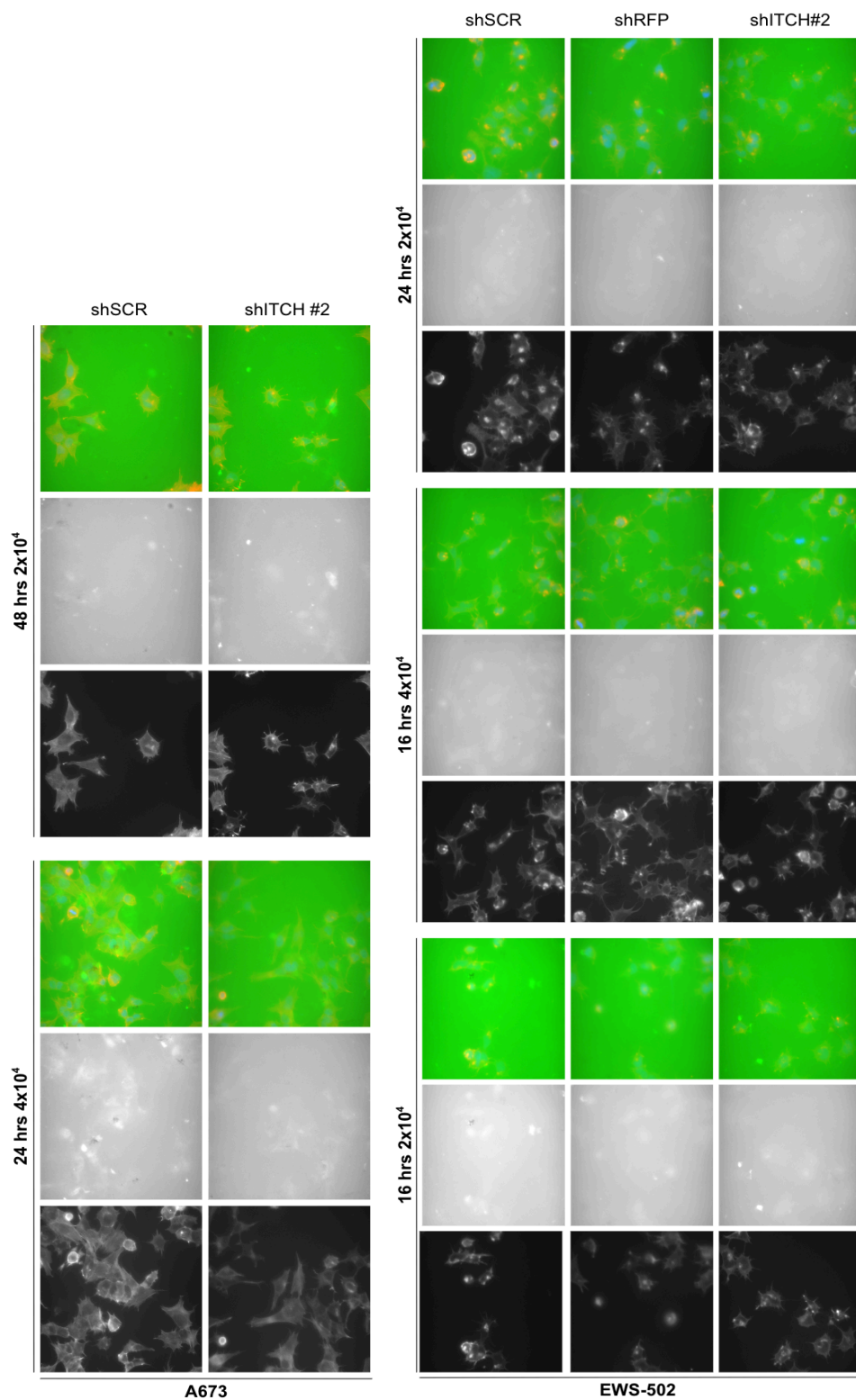
The qPCR results show the relative expression levels of ITCH in both cell lines that are transfected with various shRNAs. shGFP was not quantified since it cannot incorporate viral plasmid DNA. The 1:30 viral dilution of the EWS-502 did not have healthy samples of shRFP and shITCH #2 to quantify. Knockdown efficiency was categorized as < 0.5 .

Given these results, we decided to perform matrix degradation assays on the cells from both cell lines that were infected with shITCH #2 and exhibited sufficient ITCH knockdown. This will allow us to evaluate the role of ITCH knockdown in invadopodia formation and ECM degradation.

For the A673 cell line, two cell samples were plated: shSCR, as the control, and shITCH #2. Both samples were incubated for 24 hours with a concentration of 4×10^4 cells/well and for 48 hours with a concentration of 2×10^4 cells/well to account for cell growth. For the EWS-502 cell line, three cell samples were plated: shSCR, shRFP, and shITCH #2. All samples were incubated at varying lengths and concentrations: 2×10^4 cells/well and 4×10^4 cells/well for 16 hours, and 2×10^4 cells/well for 24 hours. These concentrations and incubation times were based on the initial degradation screenings of these cells (Figure 2.4). After incubating, the coverslips were processed and imaged as previously performed (Figure 2.5a). Additionally, the relative degradation area for the shSCR control cells was set as 1, and the values for the experimental

cells were normalized to this control (Figure 2.5b). This normalization facilitates clearer visualization of the impact of ITCH knockdown on matrix degradation across different conditions.

Figure 2.4: Matrix degradation assays for the ITCH knockdown cell samples. The A673 cells were plated in two conditions: shSCR (control) and shITCH #2 (ITCH knockdown), and the EWS-502 cells were plated in three conditions: shSCR (control), shRFP (additional control), and shITCH #2 (ITCH knockdown). All cells were plated on OG-gelatin coated coverslips and incubated for various lengths and concentrations. Nuclei were stained with DAPI and are shown in blue; F-actin were stained with phalloidin and are shown in red. OG-gelatin is shown in grayscale and areas of degradation appear as dark dots.



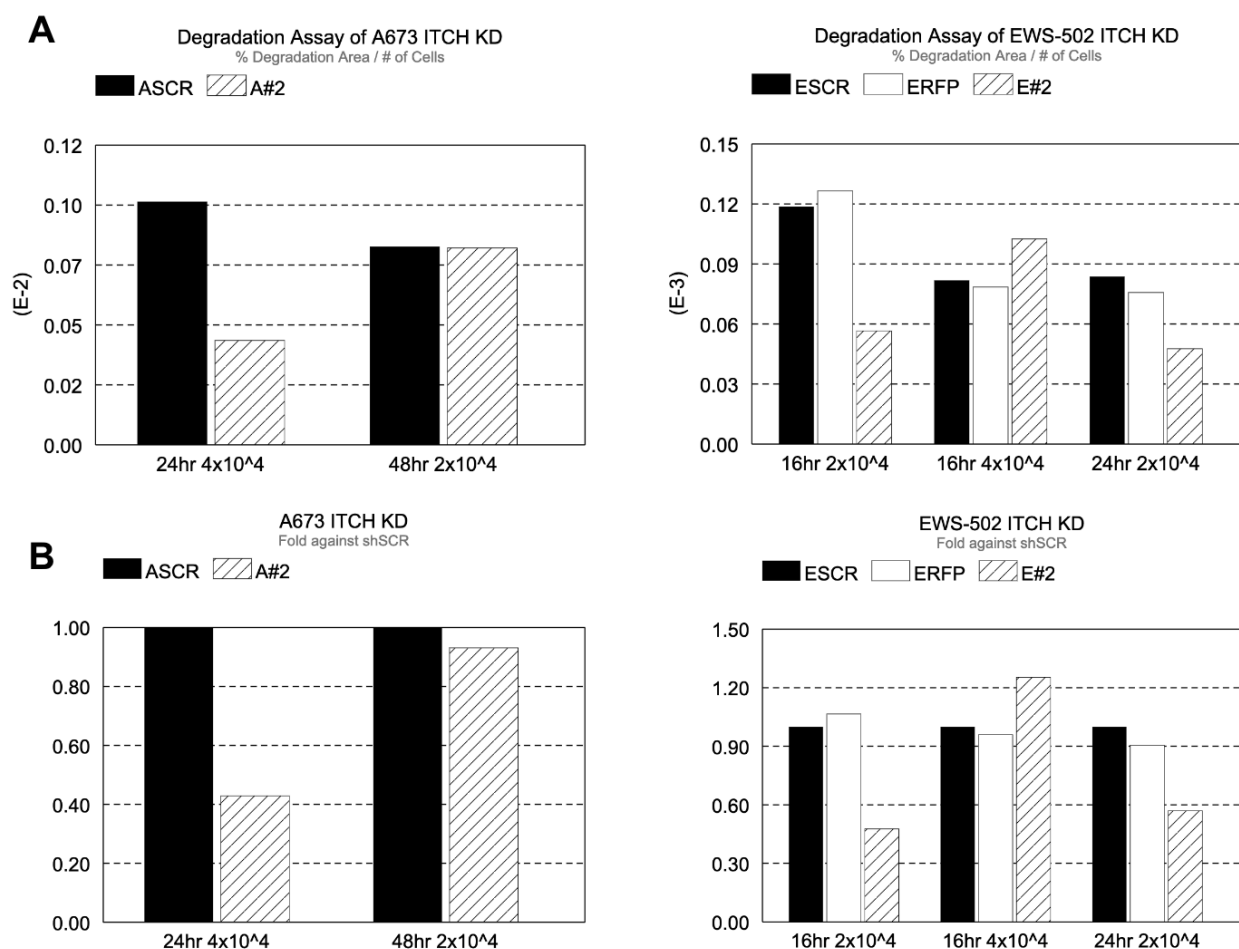


Figure 2.5: Matrix degradation assay results for A673 and EWS-502 cell lines with ITCH knockdown. (A) Quantification of matrix degradation assays for the A673 and EWS-502 cell lines plated with various shRNA constructs. Total area of degradation was measured and normalized over the number of nuclei in each field to give the relative degradation values. Note the y-axis scaled to $\times 10^{-2}$ and $\times 10^{-3}$. (B) For better visualization, the relative degradation area for the shSCR control cells was set as 1, and the values for the experimental cells were normalized to this control.

Overall, the images from the degradation assay indicated generally low degradation across all samples, resulting in limited visibility of degradation. Consequently, we decided to quantify the samples to gain a more concrete understanding of the cell lines' degradation abilities. For the A673 cell line, after 24 hours of incubation, the shITCH #2 construct exhibited approximately 60% of the degradation of the shSCR control. However, after 48 hours, shITCH #2 showed a degradation area similar to the control, suggesting potential recovery or adaptation over time. Meanwhile, for the EWS-502 cell line, at 16 hours with 2×10^4 cells/well, shITCH #2 showed a 50% reduced degradation area compared to both controls. At 16 hours with 4×10^4 cells/well, shITCH #2 surprisingly showed an increased degradation compared to both controls. Lastly, 24 hours with 2×10^4 cells/well, shITCH #2 demonstrated an approximately 40% reduction in degradation compared to both controls.

Although these results appear promising, with various samples exhibiting decreased invadopodia formation following ITCH knockdown, this experiment needs to be repeated to validate my findings in the future.

The itchy E3 ubiquitin protein ligase, otherwise known as ITCH, is an E3 ligase whose catalytic activity is stimulated by phosphorylation. This enzyme functions by attaching ubiquitin molecules to substrate proteins, marking them for degradation by the proteasome. Studies have shown that E3 ubiquitin ligases, including ITCH, play a significant role in cancer hallmark pathways such as cell cycle progression, immune evasion, inflammation, and apoptosis, with ITCH specifically promoting EMT and ECM degradation. However, its exact role in invadopodia formation has not been widely studied in various cell lines. This study focused on four new cell lines of interest: A673 (Ewing sarcoma), EWS-502 (Ewing sarcoma), TC-32 (neuroectodermal), and SK-N-MC (neuroblastoma). Upon identifying the cell lines that exhibited sufficient levels of degradation, we performed an ITCH knockdown to elucidate its role in invadopodia formation and its contribution to ECM degradation within each respective cell line.

To evaluate the ability of each new cell line to degrade the matrix and form invadopodia, we conducted and analyzed matrix degradation assays. Based on these results, the A673 and EWS-502 cell lines demonstrated the highest degradation abilities. Consequently, these cell lines were selected to assess the impact of ITCH knockdown on their degradation capabilities. Both cell lines were infected with various shRNA constructs, including shSCR (control) and shITCH #2 (ITCH knockdown). These cells were also infected with different viral concentrations of 1:2, 1:8, and 1:30. In order to fully understand and confirm the efficiency of the ITCH knockdown, we performed a qPCR analysis on the remaining healthy samples of both cell lines; the knockdown efficiency was categorized as below the threshold of 0.5. The qPCR results for the A673 cell line showed effective ITCH knockdown, particularly with the shITCH #2 construct.

Meanwhile, for the EWS-502 cell line, qPCR results indicated varying knockdown efficiencies, with only the shITCH #2 construct showing sufficient knockdown at a dilution of 1:8. The 1:30 dilution did not have any constructs that had a sufficient knockdown value; it also lacked healthy samples for shRFP and shITCH #2. Therefore, only the 1:8 dilution for the EWS-502 cell line was considered for analysis. It is important to recognize that out of all the shITCH constructs, only the shITCH #2 construct yielded an efficient ITCH knockdown.

Based on these results, we performed matrix degradation assays on cells from both lines infected with shITCH #2 and exhibiting sufficient ITCH knockdown to evaluate its role in invadopodia formation and ECM degradation. Samples from both cell lines were plated again with different cell concentrations and incubation times. For the A673 cell line, the shITCH #2 construct exhibited approximately 60% of the degradation of the shSCR control after 24 hours of incubation, indicating a significant reduction in degradation. However, the samples incubated for 48 hours showed a degradation area comparable to the control. The significant reduction in degradation at 24 hours suggests that ITCH knockdown effectively impairs the cell line's ability to form invadopodia and degrade the matrix. Meanwhile, the similar degradation levels observed at 48 hours between shITCH #2 and the control might indicate that the cells adapt or recover their degradation abilities over a prolonged period. In contrast, for the EWS-502 cell line, two cell samples demonstrated reduced degradation compared to the shSCR control: 16 hours with 2×10^4 cells/well and 24 hours with 2×10^4 cells/well; the sample incubated for 16 hours with 4×10^4 cells/well surprisingly exhibited an increase in degradation. Overall, the inconsistent degradation results at different time points and concentrations highlight the complex relationship between ITCH knockdown and invadopodia formation. Notably, the unexpected increase in

degradation at 16 hours with 4×10^4 cells/well might be due to compensatory mechanisms or experimental variability.

Unfortunately, the study encountered various obstacles that impacted the experimental outcomes. The primary challenge faced was poor growth and contamination; during the ITCH knockdown experiments, the majority of infected cell samples encountered significant issues with poor growth and contamination. This led to many unusable samples for RNA extraction and qPCR analysis, drastically reducing the number of viable samples for downstream assays. Furthermore, variability in the health and growth of the infected cell samples contributed to inconsistent experimental results.

Due to the challenges encountered with sample health and variability, the experiment needs to be repeated to validate the findings; repetition is essential to confirm the impact of ITCH knockdown on invadopodia formation and ECM degradation. Future studies should include rigorous contamination control measures and optimized growth conditions to minimize variability. In addition, utilizing multiple replicates and independent experiments will be helpful to ensure the reliability and reproducibility of the results.

CONCLUSION

Metastasis is a complex process where cancer cells spread from a primary tumor to invade surrounding tissue, enter the bloodstream, and propagate to distant organs. One way this occurs is through Epithelial-to-Mesenchymal Transition, which involves degrading and restructuring the extracellular matrix, enhancing cellular migration and invasion. A crucial element is the formation of actin-rich protrusions called invadopodia that break down the extracellular matrix, aiding cancer invasion. Notably, the E3 ubiquitin ligase ITCH, a key regulator in ubiquitination, has an unexplored role in invadopodia formation.

The first part of my research focused on investigating invadopodia formation in two previously unstudied cancer cell lines: MDA-MB-435 (melanoma) and MDA-MB-231 (highly aggressive triple-negative breast cancer). The MDA-MB-435 cell line was first assessed using various matrix degradation assays. Despite multiple experiments with different conditions (longer incubation, EGF addition, serum starvation), it showed consistently poor ECM degradation, even though Cortactin, a key invadopodia regulator, was present; this was determined using immunocytochemistry. This led to the conclusion that MDA-MB-435 is ineffective at ECM degradation, prompting a switch to the other cell line.

The MDA-MB-231 and MDA-MB-231 Oslo cell lines were studied next. Initial assays showed sufficient ECM degradation, particularly with DMSO treatment, as a positive control. However, the Oslo line performed worse than the original, leading to the decision to use only the original MDA-MB-231 line. Further Western blot analysis revealed consistent Cortactin expression but low Tks5 expression in both lines, suggesting that ITCH may influence invadopodia formation indirectly through its regulation of proteins like Tks5 and Cortactin. Again, despite multiple experiments with different conditions, the MDA-MB-231 had less

substantial degradation than anticipated; therefore, the decision was made to expand our cell line screening to include additional and more aggressive cancer cell lines.

The second part of my research focused on four new cell lines: A673 (Ewing sarcoma), EWS-502 (Ewing sarcoma), TC-32 (neuroectodermal), and SK-N-MC (neuroblastoma). Matrix degradation assays identified A673 and EWS-502 as having the highest degradation abilities. These cell lines then underwent ITCH knockdown using shRNA constructs, and qPCR confirmed efficient knockdown, particularly with the shITCH #2 construct.

Matrix degradation assays on ITCH knockdown cells revealed that A673 cells with shITCH #2 showed significant degradation reduction after 24 hours, indicating impaired invadopodia formation. However, degradation was comparable to control after 48 hours, suggesting potential adaptation. For EWS-502, inconsistent results were observed, with some samples showing reduced degradation and others showing increased degradation, indicating a complex relationship between ITCH knockdown and invadopodia formation. Due to unforeseen challenges, the study faced many issues regarding poor growth and contamination during the ITCH knockdown experiments. Many infected cell samples were unusable for RNA extraction and qPCR analysis, reducing the number of viable samples for downstream assays. Additionally, variability in the health and growth of infected samples led to inconsistent experimental results.

Based on the results, the study's outcomes did not lead to a definitive conclusion regarding specific cell line degradation ability as well as ITCH's specific role in invadopodia formation. To begin, there was a significant amount of degradation variability across all cell lines. This may have been attributed to methodological challenges, such as poor plating technique or contamination of specific cell samples, or simply human error. These issues likely contributed to inconsistent degradation results and variability in the health and growth of the

samples. Intrinsic differences between cell lines could have also been attributed to the large variability in degradation. For instance, the A673 cell line exhibited significant degradation after 48 hours, highlighting its strong invadopodia formation potential, whereas other cell lines like TC-32 and SK-N-MC showed minimal degradation, possibly due to lower intrinsic invadopodia-forming abilities.

Additionally, when comparing the final degradation assays of the A673 and EWS-502 cell lines infected with the positive control shRNA constructs to the original MDA-MB-231 cell line, the infected cell lines exhibited significantly lower degradation rates, rendering their seemingly successful ITCH knockdown unreliable. The A673 cell line infected with shSCR degraded at approximately 0.001, and the EWS-502 cell line infected with shSCR degraded at approximately 0.00012. In contrast, the MDA-MB-231 cell line in the final degradation assay degraded at a value of 0.0072, which was already deemed insufficient. Compared to the infected cell lines post-knockdown, this value is significantly higher. Thus, these results indicate that although ECM degradation in A673 and EWS-502 cell lines was relatively low, the ITCH knockdown still reduced degradation, suggesting that ITCH may be involved in the degradation process. Moving forward, these specific cell lines hold potential for further investigation to determine the precise role of ITCH in invadopodia formation and degradation.

Due to the relatively low ECM degradation of the cell lines, the exact role of ITCH in invadopodia formation was also unable to be fully determined. However, based on the results obtained from the Western blot, we came to several hypotheses; we believe that ITCH may play an indirect role in modulating the stability and activity of key proteins involved in invadopodia formation, such as Tks5 and Cortactin. Our studies found that the low Tks5 expression in the highly metastatic MDA-MB-231 and MDA-MB-435 cell lines suggest that ITCH may influence

invadopodia formation through other pathways. This indicates ITCH might be more crucial in cell lines with higher Tks5 levels, making them more dependent on ITCH for invadopodia formation and ECM degradation. Cortactin, on the other hand, showed consistent expression across tested cell lines, indicating it might not limit invadopodia formation but still plays a crucial role in actin polymerization and structural integrity. Thus, ITCH's regulation of Cortactin likely modulates invadopodia dynamics and ECM degradation efficiency rather than initiating formation.

To strengthen and build upon the findings of this study, it is extremely important to repeat the experiments with additional replicates and larger sample sizes; this will enhance the reliability and reproducibility of the results and provide a more comprehensive understanding of ITCH's role in invadopodia formation and ECM degradation. Replicates validate the consistency of observed effects across different experimental runs, thereby providing greater confidence in the conclusions, especially given the variability in degradation capabilities and challenges with cell growth and contamination. Additionally, larger sample sizes will help account for the natural heterogeneity present within and between cancer cell lines, ensuring that the observed effects of ITCH knockdown are not due to random chance or isolated instances. Lastly, to minimize inconsistencies, standardized protocols for cell plating densities, incubation times, staining techniques, and imaging methods must be established and strictly adhered to. Rigorous quality control and uniformity across all experimental steps will reduce variability and enhance result accuracy in future experiments.

As this study provides the preliminary groundwork, future research can further explore cell line degradation abilities and ITCH's role in cancer biology at the molecular level. For example, future studies should investigate the role of ITCH in cancer cell lines with higher Tks5

expression levels. Since Tks5 is critical for invadopodia formation, examining cell lines with prominent Tks5 expression may reveal a more pronounced effect of ITCH knockdown on ECM degradation and cell invasion, identifying cancer types more dependent on ITCH for metastasis and responsive to ITCH-targeted therapies. Additionally, expanding research to more diverse cancer cell types, such as primary tumor cells or its metastatic derivatives, will provide a broader understanding of ITCH's role across different cancer contexts, elucidating whether its regulatory functions are universally applicable or specific to certain subtypes. Another direction future researchers can take is by investigating ITCH's downstream targets and the specific signaling pathways it regulates; this will provide deeper insights into its role in invadopodia formation and ECM degradation. This could include identifying additional substrates ubiquitinated by ITCH and understanding how their degradation or stabilization impacts cancer cell behavior. Mechanistic studies using RNA sequencing, proteomics, and CRISPR/Cas9 gene editing can uncover detailed molecular mechanisms. Furthermore, exploring interactions between ITCH and other ubiquitin ligases, as well as their combined effects on invadopodia-associated proteins, could reveal relationships that influence cancer cell invasion and metastasis, helping to identify potential combinatory therapeutic strategies targeting multiple components of the ubiquitin-proteasome system. In summary, these expanded efforts will provide a comprehensive understanding of ITCH's multifaceted role in cancer biology, paving the way for the development of targeted therapeutic strategies aimed at inhibiting cancer metastasis.

MATERIALS & METHODS

Cell lines and culture

Cell lines used: MDA-MB-435, MDA-MB-231, MDA-MB-231 Oslo, SCC61, A673, EWS-502, SK-N-MC, TC-32, HEK293T

All cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. The serum-free media used for starvation was made with DMEM supplemented with 1% penicillin and streptomycin and without any FBS. To induce degradation formation, cells were treated with varying DMEM conditions; the conditions were the following: 0.5% FBS, 10% FBS, 2.5nM EGF, and 10nM EGF.

All cell lines were cultured at 37°C in the incubator supplied with 5% of CO₂. To passage cells, 10cm plates with cell monolayers were rinsed once with DPBS and incubated with 1 mL of 0.05% trypsin in EDTA at 37°C for 2-4 minutes. Trypsin was inactivated by adding serum media to desired final volume. If needed, the cells were spun down at 1,500rpm for 3 minutes and resuspended to desired concentrations.

Constructs

Small interfering hairpin RNA (shRNA) constructs were created. shSCR, shRFP, and shGFP were used as controls. Three shRNA lentiviral constructs against ITCH (named ITCH #1, ITCH #2, and ITCH #6) were also created.

2D-fluorescent matrix degradation assay

Matrix degradation assays were carried out as described in “Matrix Degradation Assay to Measure the Ability of Tumor Cells to Degrade Extracellular Matrix” by Fontana and Yang (2021).

Cleaning glass coverslips: Round 12mm diameter glass coverslips were incubated in 30% nitric acid overnight to remove any grease; this also helps polypeptides to adhere to the glass by enhancing polyamino acid coating. After incubating, the coverslips were washed extensively with double distilled water on a shaker, rinsing every 30 minutes for at least 4 hours. Coverslips were then transferred and stored in a clean container in 70% ethanol.

Preparing 0.2% porcine skin gelatin: 100 mg of porcine gelatin type A was measured and mixed with 50 mL of DPBS. The mixture was incubated at 37°C in a water bath for a minimum of 30 minutes and then filtered with a 0.22 µm filter into a new tube. It was stored at 4°C and is usable for up to one month.

Fluorescent gelatin coating: Tweezers were used to separate and place the coverslips on a virgin fiber tissue wiper. Once the ethanol had completely evaporated, the coverslips were transferred to a 24-well plate. In the meantime, the unlabeled 0.2% porcine skin gelatin type A and the Oregon Green (OG) 488–conjugated porcine skin gelatin were warmed in a 37°C water bath. The coverslips were coated with 500 µL of 50 µg/mL poly-L-lysine for 20 minutes, then the solution was aspirated and the coverslips were washed three times with DPBS. Then, the coverslips were incubated with 500 µL of 0.5% glutaraldehyde for 15 minutes, the

glutaraldehyde was discarded, and the coverslips were washed as before. The OG 488–gelatin solution was prepared by mixing one part fluorescent gelatin with ten parts unlabeled porcine gelatin, and the solution was warmed in a 37°C water bath. 70% ethanol was sprayed on a plastic lid, a piece of parafilm was attached, and 20 µL of OG 488–gelatin solution was pipetted onto the parafilm. A needle and tweezers were used to gently invert the coverslips over the gelatin drop, and they were incubated for 5 minutes in the dark. The coverslips were carefully transferred to a 24-well plate, washed as before, and the coating steps were repeated for all desired coverslips. The coverslips were incubated with 500 µL of 5 mg/mL sodium borohydride for 15 minutes with gentle shaking, then the solution was discarded and the coverslips were washed three times with DPBS. Finally, 1 mL of the complete medium appropriate for the specific cell type was added to the 24-well plate, and the coverslips were incubated at 37°C for 1 hour before seeding the cells.

Seeding cells: Cells were trypsinized and suspended as mentioned above. The number of cells were counted using a hemocytometer.

Fluorescent gelatin degradation assay: The target cells were seeded onto gelatin-coated coverslips in a 24-well plate and incubated in a tissue culture incubator at 37°C with 5% CO₂ for 16-48 hours. Thirty minutes before the end of the incubation period, PBS and 4% paraformaldehyde (PFA) were warmed in a 37°C water bath. At the end of the incubation, the media from each well was aspirated and the cells were washed with 1 mL of PBS. The cells were fixed by adding 1 mL of pre-warmed 4% PFA to each well and incubating the plate for 15 minutes at 37°C. The PFA was discarded, and the cells were washed three times with PBS. The

cells were then permeabilized by adding 1 mL of 0.05% Triton X-100 and incubating for 10 minutes in the dark, followed by another PBS wash. To visualize invadopodia, the coverslips were incubated with 300 μ L of Alexa Fluor 546 Phalloidin (diluted 1:1000 in 1% BSA in PBST) for 1 hour in the dark, then washed again. Up to three coverslips were mounted on one glass microscope slide using a needle and tweezers to gently place them, with excess PBS absorbed by touching the edge with a tissue wiper. The coverslips were inverted onto 5 μ L of mounting medium containing DAPI, excess medium was aspirated, and the edges were fixed and sealed with nail polish. The slides were then stored at 4°C in the dark.

Imaging and quantification: The slides were imaged using a conventional fluorescent microscope to detect nuclei (DAPI-stained), the gelatin matrix (Oregon green 488-stained), and the F-actin cytoskeleton (Alexa Fluor 546 Phalloidin-stained). Using 40x magnification, at least 30 fields per coverslip were randomly imaged to quantify the degradation area and invadopodia formation. Gelatin degradation appeared as dark spots over the green background. The images were analyzed with ImageJ to quantify invadopodia function. DAPI and fluorescent gelatin images were opened and stacked in ImageJ, converted to 8-bit, and adjusted for brightness and contrast. The degradation area percentage was quantified by thresholding the fluorescent gelatin image and analyzing particles to determine the area fraction. The number of cells per field was counted by thresholding the DAPI image and analyzing particles with adjusted size parameters. The final gel degradation index was calculated as the average area fraction normalized to the total cell number across all analyzed fields, followed by appropriate statistical analysis.

Immunocytochemistry assay

Glass coverslip preparation, cell seeding, and 0.2% porcine gelatin preparation was performed in the same manner as the degradation assay.

Porcine gelatin coating: Tweezers were used to separate and place the coverslips on a virgin fiber tissue wiper. Once the ethanol had completely evaporated, the coverslips were transferred to a 24-well plate. In the meantime, the unlabeled 0.2% porcine skin gelatin type A and the Oregon Green (OG) 488–conjugated porcine skin gelatin were warmed in a 37°C water bath. The coverslips were coated with 500 µL of 50 µg/mL poly-L-lysine for 20 minutes, then the solution was aspirated and the coverslips were washed three times with DPBS. Then, the coverslips were incubated with 500 µL of 0.5% glutaraldehyde for 15 minutes, the glutaraldehyde was discarded, and the coverslips were washed as before. 500 µL of 0.2% porcine gelatin was directly added to the coverslips and then incubated for 10 minutes at 37°C. The gelatin mixture was then aspirated and the coverslips were once again washed three times with PBS. As earlier, the coverslips were incubated with 500 µL of 5 mg/mL sodium borohydride for 15 minutes with gentle shaking, then the solution was discarded and the coverslips were washed three times with DPBS. Finally, 1 mL of the complete medium appropriate for the specific cell type was added to the 24-well plate, and the coverslips were incubated at 37°C for 1 hour before seeding the cells.

Fixing and staining: 4% paraformaldehyde supplemented with krebs and sucrose fixative (4% PKS) was used as a fixative agent and was heated to 37°C in a water bath. Samples were washed once with PBS and fixed with 500 µL of 4% PKS for 15 minutes at 37°C. They were

then washed three times with PBS. After the washes, samples were blocked in 1% BSA in PBST for 1 hour at room temperature. The primary antibody of interest was diluted in the blocking solution, and cells were incubated in the diluted antibody in 1% BSA in PBST (using 35 μ L) in a humidified chamber overnight at 4°C. The primary antibody was washed off with the blocking solution three times, followed by a secondary blocking for 1 hour at room temperature to reduce background. Cells were then incubated with the diluted secondary antibody and Phalloidin in 1% BSA in PBST in a humidified chamber for 1 hour at room temperature. Wells were washed with PBS to remove BSA residues, followed by three washes with PBS. Coverslips were stained with DAPI, mounted on slides using 4.5 μ L of mounting media, and sealed with nail polish. Coverslips were cleaned with Fisher Optical Cleaner to remove PBS residue and excess nail polish, using 50 μ L for each coverslip and aspirating with a 100 μ L tip. The process was repeated if necessary. Finally, the samples were imaged with a fluorescent microscope.

The Tks5 mouse primary antibody was used at 1:100 dilution. The Alexa fluor 488 secondary antibody was used at 1:200 dilution.

Imaging: The slides were imaged using a conventional fluorescent microscope to detect nuclei (DAPI-stained), the gelatin matrix (Alexa fluor 488-stained), and the F-actin cytoskeleton (Alexa Fluor 546 Phalloidin-stained). Using 40x magnification, at least 30 fields per coverslip were randomly imaged to quantify the degradation area and invadopodia formation. Tks5 should have appeared as white dots over the Alexa fluor 488 field.

Western blot

Protein extraction: Cells were cultured for two days to reach confluency. Cells were rinsed in cold 1X PBS, aspirated, and 100 μL of lysis buffer was added to each dish. The lysis buffer was a radio-immunoprecipitation assay buffer (RIPA) supplemented with a 1:200 dilution of protease inhibitor cocktail (PIC) and 1:100 dilution of sodium orthovanadate (Na_3VO_4); a 1:100 dilution of phenylmethylsulfonyl fluoride (PMSF) could also optionally be included. Whole lysate was scraped down with a spatula, collected in tubes, and incubated in ice for 40 minutes. Samples were then sonicated 4 seconds on and 3 seconds off for 16 seconds total and spun in a 4°C cold centrifuge for 15 minutes at 15,000 rpm. The sample supernatants were then collected and transferred to fresh cold tubes.

Protein quantification: Protein concentration was determined using the DC protein assay (Bio-Rad). 100 μL of reagent A, an alkaline copper tartrate solution, and 2 μL of reagent S were prepared according to the number of samples intended to quantify. 2 μL of stock protein was added to each tube, along with varying amounts of BSA stock solution (10 mg/mL) to create a standard curve; the amounts were 0 μL , 1 μL , 2 μL , and 4 μL . 800 μL of reagent B was then added to each sample and incubated at room temperature for 15 minutes.

Protein concentration was measured using a spectrophotometer set to Lowry mode. The samples were transferred into the cuvettes and protein concentrations read at 750 nm. The ideal regression value was between 0.9 and 1.

Running the Western blot: Proteins were denatured by boiling for 10 minutes with a 1:20 dilution of 1M dithiothreitol (DTT) and 4X Laemmli loading buffer. Samples were loaded onto a pre-made SDS-PAGE gel with a MOPS buffer and ran at 100V and 200 mA for 40 minutes or until the loading dye reached the bottom. Protein was then transferred onto a PVDF membrane by creating a Western blot transfer sandwich and ran in cold temperatures for 60 minutes at 350 mA.

The membrane was rinsed once with PBS supplemented with 1% tween (PBST) and blocked with 2.5% BSA in PBS for at least 15 minutes on the shaker. The primary antibody was added to 5% milk in PBS and incubated overnight in the cold room. After, the membrane was washed three times with PBST and incubated with the appropriate secondary HRP conjugated antibody in 5% milk in PBS for 1 hour on the shaker. Lastly, the membrane was washed three times again with PBST.

The membrane was visualized with an ECL Plus Western Blotting detection kit and exposed using a BIO-RAD fluorescence microscope.

The Tks5 mouse primary antibody was used at 1:1000 dilution. The Cortactin mouse primary antibody was used at 1:1000 dilution. The HRP conjugated secondary antibody was used at 1:14,000 dilution for both aforementioned primary antibodies. The GAPDH rabbit primary antibody was used at 1:10,000 dilution, and the HRP conjugated secondary antibody for that was used at 1:20,000 dilution.

ITCH knockdown

Lentiviral production and infection: HEK293T cells were seeded onto 4cm, 6-well dishes in serum media. Cell lines were generated via infection of target cells using lentiviruses produced by transfection of the viral vector with 0.9 µg of packaging (pCMVΔ8.2R) and 0.1 µg of envelope (pVSV-G) plasmids into HEK293T cells using TransIT-LT1 (Mirus). 2 µg of DNA was added to the mix and incubated at 37°C overnight. 18 hours post-transfection, the media of the transfected cells were changed and the target cells were seeded at concentrations such that they will be 20% confluent the following day. The A673 cells were seeded at 36×10^4 cells and the EWS-502 cells were seeded at 32×10^4 cells. Viral supernatants were harvested at 48 and 72 hours and filtered using a 0.45 µm filter and dripped onto target cells. Protamine sulfate (6 µg/mL) was added to the viral supernatant in a 1:1000 dilution to enhance the efficiency of viral transduction. Cells were then selected with a 1:4000 dilution of puromycin and maintained in the incubator as before.

RNA extraction: RNA extraction and purification was performed using the NucleoSpin RNA mini kit. Cells were cultured for two days to confluency and rinsed in PBS before harvesting. Cells were lysed using a mixture of 350 µL RA1 solution and 3.5 µL of β-mercaptoethanol before being transferred into a filter and spun at 11,000 g for 1 minute. The filter was discarded and 350 µL of 70% ethanol was added to before being spun again at 11,000 g for 30 seconds. To desalt the membrane, 350 µL of membrane desalting buffer (MBD) was added and centrifuged at 11,000 g for 1 minute to dry the membrane. A DNase reaction mixture was made using 10 µL of reconstituted rDNase and 90 µL of reaction buffer for DNase per sample, and 95 µL of the reaction mixture was added to the column membrane. The samples

were incubated at room temperature for 15 minutes before being washed and dried three times, being replaced each time in a new 2mL collection tube. In the first drying step, 200 μ L of RAW2 buffer was added and the sample was spun for 30 seconds at 11,000 g before being moved to a new collection tube. In the second drying step, 600 μ L of RA3 buffer was added, and the sample was spun for 30 seconds at 11,000 g again. In the last drying step, 250 μ L of RA3 buffer was added, and the sample was spun for 2 minutes at 11,000 g. In order to fully dry the membrane, the samples were spun at the highest speed allowed by the centrifuge for 2 minutes. The RNA samples were finally eluted in 40 μ L of RNase-free H₂O and RNA concentration was quantified using a nano-photometer.

cDNA generation: cDNA was synthesized from RNA. The reaction mix consisted of 10X RT buffer, 10X reverse transcriptase, 25X NTP 100mM, MultiScribe reverse transcriptase, and RNase water. 2 μ g of RNA was added to the reaction mixture, and the reaction was run in a BIO-RAD thermal cycler at the following cycles: 10 minutes at 25°C, 60 minutes at 37°C, and 5 seconds at 85°C. The cycle was repeated for a second time, and the final 20 μ L cDNA product was diluted with 180 μ L of MQ water.

Quantitative Real-Time PCR: qRT-PCR was performed using the SYBR® Green PCR Master Mix (Applied Biosystems). The qRT-PCR mixed consisted of the SYBR Green 1 Dye, 10 μ M forward primer, 10 μ M reverse primer, a reference dye, and water. 6 μ L of the mixture were mixed with 4 μ L of cDNA. Results were recorded and calculated on Google Sheets.

REFERENCES

- Abadie, A., Besançon, F., & Wietzerbin, J. (2004). Type I interferon and TNF α cooperate with type II interferon for TRAIL induction and triggering of apoptosis in SK-N-MC EWING tumor cells. *Oncogene*, 23(28), 4911–4920. <https://doi.org/10.1038/sj.onc.1207614>
- Alblazi, K. M. O., & Siar, C. H. (2015). Cellular Protrusions - Lamellipodia, Filopodia, Invadopodia and Podosomes - and their Roles in Progression of Orofacial Tumours: Current Understanding. *Asian Pacific Journal of Cancer Prevention*, 16(6), 2187–2191. <https://doi.org/10.7314/APJCP.2015.16.6.2187>
- Ayala, I., Baldassarre, M., Caldieri, G., & Buccione, R. (2006). Invadopodia: A guided tour. *European Journal of Cell Biology*, 85(3), 159–164. <https://doi.org/10.1016/j.ejcb.2005.09.005>
- Bailey, K. M., Airik, M., Krook, M. A., Pedersen, E. A., & Lawlor, E. R. (2016). Micro-Environmental Stress Induces Src-Dependent Activation of Invadopodia and Cell Migration in Ewing Sarcoma. *Neoplasia*, 18(8), 480–488. <https://doi.org/10.1016/j.neo.2016.06.008>
- Bertier, L., Hebbrecht, T., Mettepenningen, E., De Wit, N., Zwaenepoel, O., Verhelle, A., & Gettemans, J. (2018). Nanobodies targeting cortactin proline rich, helical and actin binding regions downregulate invadopodium formation and matrix degradation in SCC-61 cancer cells. *Biomedicine & Pharmacotherapy*, 102, 230–241. <https://doi.org/10.1016/j.biopha.2018.03.064>
- Christofori, G. (2006). New signals from the invasive front. *Nature*, 441(7092), 444–450. <https://doi.org/10.1038/nature04872>
- Coopman, P. J., Do, M. T., Thompson, E. W., & Mueller, S. C. (1998). Phagocytosis of cross-linked gelatin matrix by human breast carcinoma cells correlates with their invasive capacity. *Clinical Cancer Research*, 4(2), 507–515.
- Diaz, B., Shani, G., Pass, I., Anderson, D., Quintavalle, M., & Courtneidge, S. A. (2009). Tks5-Dependent, Nox-Mediated Generation of Reactive Oxygen Species Is Necessary for Invadopodia Formation. *Science Signaling*, 2(88). <https://doi.org/10.1126/scisignal.2000368>

- Fidler, I. J. (1989). Origin and biology of cancer metastasis. *Cytometry*, 10(6), 673–680. <https://doi.org/10.1002/cyto.990100602>
- Fontana, R., Mestre-Farrera, A., & Yang, J. (2024). Update on Epithelial-Mesenchymal Plasticity in Cancer Progression. *Annual Review of Pathology: Mechanisms of Disease*, 19 (Volume 19, 2024), 133–156. <https://doi.org/10.1146/annurev-pathmechdis-051222-122423>
- Fontana, R., & Yang, J. (2021). Matrix Degradation Assay to Measure the Ability of Tumor Cells to Degrade Extracellular Matrix. *Methods in Molecular Biology (Clifton, N.J.)*, 2294, 151–163. https://doi.org/10.1007/978-1-0716-1350-4_11
- Gallagher, E., Gao, M., Liu, Y.-C., & Karin, M. (2006). Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. *Proceedings of the National Academy of Sciences of the United States of America*, 103(6), 1717–1722. <https://doi.org/10.1073/pnas.0510664103>
- Geiger, T. R., & Peeper, D. S. (2009). Metastasis mechanisms. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1796(2), 293–308. <https://doi.org/10.1016/j.bbcan.2009.07.006>
- Hegedüs, L., Cho, H., Xie, X., & Eliceiri, G. L. (2008). Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. *Journal of Cellular Physiology*, 216(2), 480–485. <https://doi.org/10.1002/jcp.21417>
- Jacob, A., & Prekeris, R. (2015). The regulation of MMP targeting to invadopodia during cancer metastasis. *Frontiers in Cell and Developmental Biology*, 3. <https://doi.org/10.3389/fcell.2015.00004>
- Jeannot, P., & Besson, A. (2020). Cortactin function in invadopodia. *Small GTPases*, 11(4), 256–270. <https://doi.org/10.1080/21541248.2017.1405773>
- Meng, D., Meng, M., Luo, A., Jing, X., Wang, G., Huang, S., Luo, M., Shao, S., Zhao, X., & Liu, R. (2019). Effects of VEGFR1+ hematopoietic progenitor cells on pre-metastatic niche formation and in vivo metastasis of breast cancer cells. *Journal of Cancer Research and Clinical Oncology*, 145(2), 411–427. <https://doi.org/10.1007/s00432-018-2802-6>

- Pagani, A., Fischer-Colbrie, R., Sanfilippo, B., Winkler, H., Cerrato, M., & Bussolati, G. (1992). Secretogranin II expression in Ewing's sarcomas and primitive neuroectodermal tumors. *Diagnostic Molecular Pathology: The American Journal of Surgical Pathology, Part B*, 1(3), 165–172.
- Paz, H., Pathak, N., & Yang, J. (2014). Invading one step at a time: The role of invadopodia in tumor metastasis. *Oncogene*, 33(33), 4193–4202. <https://doi.org/10.1038/onc.2013.393>
- Pedersen, N. M., Wenzel, E. M., Wang, L., Antoine, S., Chavrier, P., Stenmark, H., & Raiborg, C. (2020). Protrudin-mediated ER–endosome contact sites promote MT1-MMP exocytosis and cell invasion. *Journal of Cell Biology*, 219(8), e202003063. <https://doi.org/10.1083/jcb.202003063>
- Pickart, C. M. (2001). Mechanisms Underlying Ubiquitination. *Annual Review of Biochemistry*, 70(Volume 70, 2001), 503–533. <https://doi.org/10.1146/annurev.biochem.70.1.503>
- Renshaw, S. (2016). Immunohistochemistry and Immunocytochemistry. In S. Renshaw (Ed.), *Immunohistochemistry and Immunocytochemistry: Essential Methods* (pp. 35–102). John Wiley & Sons, Ltd. <https://doi.org/10.1002/9781118717769.ch3>
- Salah, Z., Itzhaki, E., & Aqeilan, R. I. (2014). The ubiquitin E3 ligase ITCH enhances breast tumor progression by inhibiting the Hippo tumor suppressor pathway. *Oncotarget*, 5(21), 10886–10900.
- Sampson, C., Wang, Q., Otkur, W., Zhao, H., Lu, Y., Liu, X., & Piao, H. (2023). The roles of E3 ubiquitin ligases in cancer progression and targeted therapy. *Clinical and Translational Medicine*, 13(3), e1204. <https://doi.org/10.1002/ctm2.1204>
- Sankar, S., Theisen, E. R., Bearss, J., Mulvihill, T., Hoffman, L. M., Sorna, V., Beckerle, M. C., Sharma, S., & Lessnick, S. L. (2014). Reversible LSD1 Inhibition Interferes with Global EWS/ETS Transcriptional Activity and Impedes Ewing Sarcoma Tumor Growth. *Clinical Cancer Research*, 20(17), 4584–4597. <https://doi.org/10.1158/1078-0432.CCR-14-0072>
- Saykali, B. A., & El-Sibai, M. (2014). Invadopodia, Regulation, and Assembly in Cancer Cell Invasion. *Cell Communication & Adhesion*, 21(4), 207–212. <https://doi.org/10.3109/15419061.2014.923845>

- Schoumacher, M., Goldman, R. D., Louvard, D., & Vignjevic, D. M. (2010). Actin, microtubules, and vimentin intermediate filaments cooperate for elongation of invadopodia. *Journal of Cell Biology*, 189(3), 541–556. <https://doi.org/10.1083/jcb.200909113>
- Siegel, R. L., Giaquinto, A. N., & Jemal, A. (2024). Cancer statistics, 2024. *CA: A Cancer Journal for Clinicians*, 74(1), 12–49. <https://doi.org/10.3322/caac.21820>
- Takkunen, M., Hukkanen, M., Liljeström, M., Grenman, R., & Virtanen, I. (2010). Podosome-like structures of non-invasive carcinoma cells are replaced in epithelial-mesenchymal transition by actin comet-embedded invadopodia. *Journal of Cellular and Molecular Medicine*, 14(6b), 1569–1593. <https://doi.org/10.1111/j.1582-4934.2009.00868.x>
- Teicher, B. A., Bagley, R. G., Rouleau, C., Kruger, A., Ren, Y., & Kurtzberg, L. (2011). Characteristics of human Ewing/PNET sarcoma models. *Annals of Saudi Medicine*, 31(2), 174–182. <https://doi.org/10.4103/0256-4947.7820>
- Thiery, J. P., & Sleeman, J. P. (2006). Complex networks orchestrate epithelial–mesenchymal transitions. *Nature Reviews Molecular Cell Biology*, 7(2), 131–142. <https://doi.org/10.1038/nrm1835>
- Weaver, A. M. (2006). Invadopodia: Specialized Cell Structures for Cancer Invasion. *Clinical & Experimental Metastasis*, 23(2), 97–105. <https://doi.org/10.1007/s10585-006-9014-1>
- Yamaguchi, H. (2012). Pathological roles of invadopodia in cancer invasion and metastasis. *European Journal of Cell Biology*, 91(11), 902–907. <https://doi.org/10.1016/j.ejcb.2012.04.005>
- Yamamoto, H., Sutoh, M., Hatakeyama, S., Hashimoto, Y., Yoneyama, T., Koie, T., Saitoh, H., Yamaya, K., Funyu, T., Nakamura, T., Ohyama, C., & Tsuboi, S. (2011). Requirement for FBP17 in Invadopodia Formation by Invasive Bladder Tumor Cells. *The Journal of Urology*. <https://doi.org/10.1016/j.juro.2010.12.027>