# UC San Diego

## UC San Diego Electronic Theses and Dissertations

### Title

Analysis of an Inducible-Twist1 Breast Cancer Mouse Model to Investigate Metastasis Dormancy

### Permalink

https://escholarship.org/uc/item/0pv5c0sn

### Author

Lee, Tiffany

### Publication Date

2018

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA SAN DIEGO

# Analysis of an Inducible-Twist1 Breast Cancer Mouse Model to Investigate Metastasis Dormancy

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Tiffany Lee

Committee in charge:

Professor Jing Yang, Chair
Professor Gen-Sheng Feng, Co-Chair
Professor Xin Sun

2018

Copyright

Tiffany Lee, 2018

All rights reserved.

The Thesis of Tiffany Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-Chair

_____

Chair

University of California San Diego

2018

TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| BM | Basement Membrane |
| CTC | Circulating Tumor Cells |
| Doxy | Doxycycline |
| DTCs | Disseminated Tumor Cells |
| ECM | Extracellular Matrix |
| EMT | Epithelial-Mesenchymal Transition |
| FACS | Fluorescence-Activated Cell Sorting |
| GFP | Green Fluorescence Protein |
| IF | Immunofluorescence |
| MET | Mesenchymal-Epithelial Transition |

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Jing Yang for her support as the chair of my committee. She has provided invaluable guidance throughout the master's program.

I would also like to acknowledge my mentors Haeyun Jung and Kay Yeung for their expert advice and support.

The results, in part, have unpublished material from my co-authors Haeyun Jung and Kay Yeung. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

Analysis of an Inducible-Twist1 Breast Cancer Mouse Model to Investigate Metastasis Dormancy

by

Tiffany Lee

Master of Science in Biology

University of California San Diego, 2018

Professor Jing Yang, Chair
Professor Gen-Sheng Feng, Co-Chair

Most breast cancer-related deaths are due to distant metastasis that occur years after successful tumor removal and treatment. Tumor metastasis is a multi-step process in which primary tumors lose cell polarity and undergo dissemination by Epithelial-Mesenchymal Transition (EMT). The disseminated tumor cells circulate throughout the body and often remain in a dormant state after reaching distant organs. Once the microenvironment

becomes suitable, the cells undergo Mesenchymal-Epithelial Transition (MET) and exit dormancy, which results in macrometastases. Previous studies show that a transcription factor Twist1 plays a key role in inducing EMT and promoting tumor metastasis. However, the molecular mechanisms of metastasis are not well understood due to the lack of an in vivo mouse model that can monitor disseminated tumor cells. Therefore, we established a Twist1-inducible breast cancer mouse model that utilizes fluorescent markers to visualize and characterize disseminated tumor cells at various stages of metastasis.

# INTRODUCTION

## Breast Cancer

Cancer is a collection of diseases in which cells obtain the ability to grow uncontrollably and the spread of these uncontrolled cells may result in death. In United States, about 1 million new cancer cases will be diagnosed in 2018 and about 600,000 are expected to die of cancer in 2018; therefore making cancer the second leading cause of death (American Cancer Society, 2018). Currently, the four most common cancer types are lung, colorectal, breast, and prostate (American Cancer Society, 2018).

For women, breast cancer is the most commonly diagnosed cancer and is the second-leading cause of death following lung cancer (American Cancer Society, 2018). Breast cancer is the uncontrolled growth of breast cells that commonly results as a lump or mass in the breast. Although the development of early detection methods and surgical procedures have increased treatment prognosis for localized breast cancer, 90% of breast cancer-related deaths are due to metastasis (Fidler, 2003). Breast cancer can metastasize to several locations, including the lung, bone, liver, and brain, and mechanisms of breast cancer metastasis remain largely unknown (American Cancer Society, 2018).

## Metastasis

Metastasis is the spread of cancer from one location to another. Metastasis is a multi-step process that first involves primary epithelial tumor cells gaining the ability to invade into the extracellular matrix (ECM), intravasate into the blood and lymphatic circulatory system, extravasate out of the circulatory system, establish at secondary site, and eventually proliferate into macrometastastic lesions (Lambert *et al*., 2017).

During development, epithelial cells form into sheets of cells that are tightly held by cell junctions such as tight junctions, adherens junctions, gap junctions, desmosomes, and hemidesmosomes (Yap et al., 1997). These cell junctions function to maintain cell to cell adhesion among the sheets of epithelial cells. The sheets of epithelial cells anchor to the basement membrane to establish apical basal polarity, therefore maintaining lateral movement and preventing invasion into the ECM (Yap et al., 1997). However, tumor cells can go through the process termed Epithelial-Mesenchymal Transition (EMT) to lose epithelial traits and become more mesenchymal, thereby gaining the ability to breach the basement membrane to locally invade and to enter the systemic circulation.

The tumor cells that intravasate and migrate in the blood are termed as circulating tumor cells (CTCs). CTCs harbor the characteristics of the cancer disease and can be collected from the blood, which is less invasive than tumor biopsies. Therefore, in the recent years many studies have suggested that CTCs can serve as a promising biomarker for early detection of metastasis, evaluation of treatment responses, and prediction of therapy resistance (Mousavi-Harami et al., 2014). Furthermore, studies have found that higher CTC count correlated with metastatic breast cancer disease and worse prognosis for breast cancer patients (Cristofanilli et al., 2004).

The final step of metastasis involves CTCs extravasating out of the circulatory system and establishing at the distant organ for metastatic growth. However, most of the tumor cells that enter circulation are either unable to survive in circulation or unable to survive in the new distant organ microenvironment (Dasgupta et al., 2017). Tumor cells that are not eliminated at the new environment enter growth arrest called tumor dormancy as single disseminated tumor cells (DTCs) due to the inability to adapt to the new microenvironment (Lambert *et al*., 2017). Tumor dormancy contributes to the recurrence and metastatic disease years after patients had received

successful removal of primary tumors and treatments. Current treatment such as immune surveillance and chemotherapy are unable to target and effectively eliminate dormant DTCs since these cells are not proliferating (Gao et al., 2017). Therefore, dormant DTCs can persist in the body for years until the microenvironment is suitable again for re-proliferation into macrometastases. However, the mechanisms of tumor dormancy and how tumor cells exit dormancy are unknown. It is thus crucial to under the mechanisms to discover new therapeutic approaches in eliminating dormant DTCs.

**Epithelial-Mesenchymal Transition (EMT) and Mesenchymal-Epithelial Transition (MET)**

Epithelial-Mesenchymal Transition (EMT) is important for the initial step of metastasis in which tumor cells gain the ability to disseminate and migrate. Epithelial cells are stationary and unable to cross into the ECM due to cell to cell adhesion and apical-basal polarity. However, during EMT, epithelial tumor cells lose polarity and transition into elongated mesenchymal cells that can invade the ECM and enter systemic circulation. EMT was first identified as a program occurring in embryonic development, but many studies have since recognized EMT as an important process in tumor dissemination and metastasis (Yang *et al*., 2008). EMT is also implicated in the wound healing process since cells need to have the ability to migrate to the wound site to repair the epithelium (Weinberg, 2007). The similarities between cell movement during metastasis and development suggests tumor cells can hijack this EMT program to initiate cell morphology change to gain the ability to invade and migrate.

Previous studies have indicated that circulating tumor cells were not strictly mesenchymal phenotype but expressed both epithelial and mesenchymal markers, suggesting plasticity in the EMT process (Santamaria et al., 2017).

Furthermore, the tumor cells that exit dormancy and regain proliferation at a secondary site are no longer mesenchymal but have epithelial characteristics, suggesting the reverse process of EMT called Mesenchymal-Epithelial Transition (MET) is important for metastatic growth (Yao et al., 2011).

Twist1 is one of the many EMT-activating transcription factors that play a key role in tumor metastasis. Twist1 is a basic helix-loop-helix transcription factor that has been found to be overexpressed in cancers such as breast, prostate, gastric, and squamous cell carcinoma (SCC) (Qin et al., 2012). Clinical data has shown that Twist1 expression is correlated with increase metastasis and poor survival in human breast cancer patients (Eckert *et al*., 2011). Furthermore, Previous studies from our has found that induction of Twist1 expression is essential for initiating EMT and tumor metastasis (Yang et al., 2004). Yet, Twist1 expression needs to be turned off for disseminated cells to undergo the reverse process MET to regain proliferation and form macrometastases (Tsai *et al*., 2012). EMT is therefore crucial during the initial stages of metastasis in which tumor cells gain the ability to invade and migrate while MET is important for the later stages of metastasis for formation of macrometastases.

**Specific aims**

One of the major obstacles of understanding the mechanisms of EMT, tumor dormancy, and metastasis is the lack of current in vivo mouse models that can effectively control cell dissemination and track tumor cells throughout the stages of metastasis. To understand what microenvironment factors are influencing the changes between the stages of metastasis, there needs to be a way to control when EMT occurs. Furthermore, there needs to be a way to visualize and track tumor cells that disseminated from the primary tumor.

Therefore, we aim to establish a novel Twist1-inducible mouse model that utilizes doxycycline to control cell dissemination and fluorescence to visualize tumor cells during metastasis. This model will serve as an important tool for understanding the mechanisms regulating tumor dormancy and metastasis; therefore providing insights to eradicate dormant metastatic tumor cells.

# RESULTS

## Generation of Twist1 Inducible Breast Cancer Tumor Dormancy Mouse Model

Previous studies from our lab have demonstrated that Twist1 is necessary for initiating EMT and tumor metastasis (Yang et al., 2004). However, one of the major obstacles in understanding the mechanisms of EMT, tumor dormancy, and metastasis has been the lack of mouse models that can monitor disseminated tumor cells in a controlled fashion. To address this issue, we generated a triple transgenic mice ROSA-rtTA:TetON-Twist1:TetON-GFP (RTG) that has Twist1 and GFP controlled under the same TetOP promoter when doxycycline is present (Figure 1). GFP will serve as the fluorescent marker for Twist1 expression when doxycycline is administered, which allows us to induce and monitor disseminated tumor cells in a controlled manner.

To establish a mouse model that emulates the multiple stages of metastasis, the primary mammary tumors were generated by delivering Her2-tdTomato lentivirus into the mammary glands via intraductal injections in RTG mice (Figure 2). Once mammary tumors reached 1cm, the tumors were transplanted from RTG mice into FVB mice to efficiently generate primary tumors in a large number of mice. Furthermore, when doxycycline is administered, the expression of Twist1/GFP will only be targeted in the tumors rather than in the whole body. Once tumors in FVB mice reached 1cm, the mice were given doxycycline in drinking water for three weeks to induce Twist1 expression and dissemination. The tumors were surgically removed, blood samples were collected, and the mice were randomly divided into two groups. The first group will receive continuous doxycycline treatment to maintain Twist1 expression and EMT to remain in a dormant state. The second group will be withdrawn from doxycycline to

stop Twist1 expression and induce MET. After 2-3 months, the lungs were harvested to analyze for metastatic lesions.

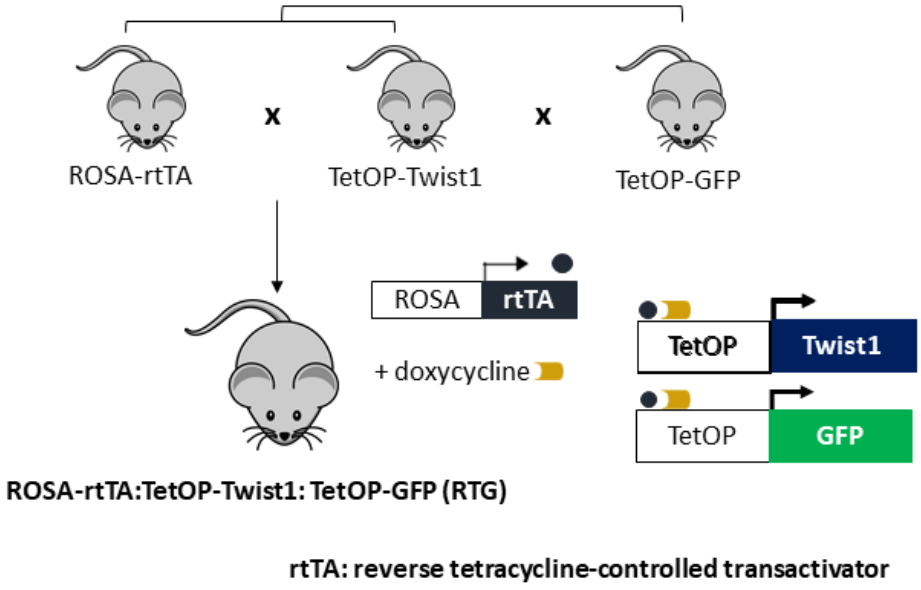


**Figure 1. Triple Transgenic RTG Mouse Construct**
Schematic of the generation of triple transgenic ROSA-rtTA:TetOP-Twist1:TetOP-GFP (RTG) mouse that is Twist1 inducible by doxycycline.

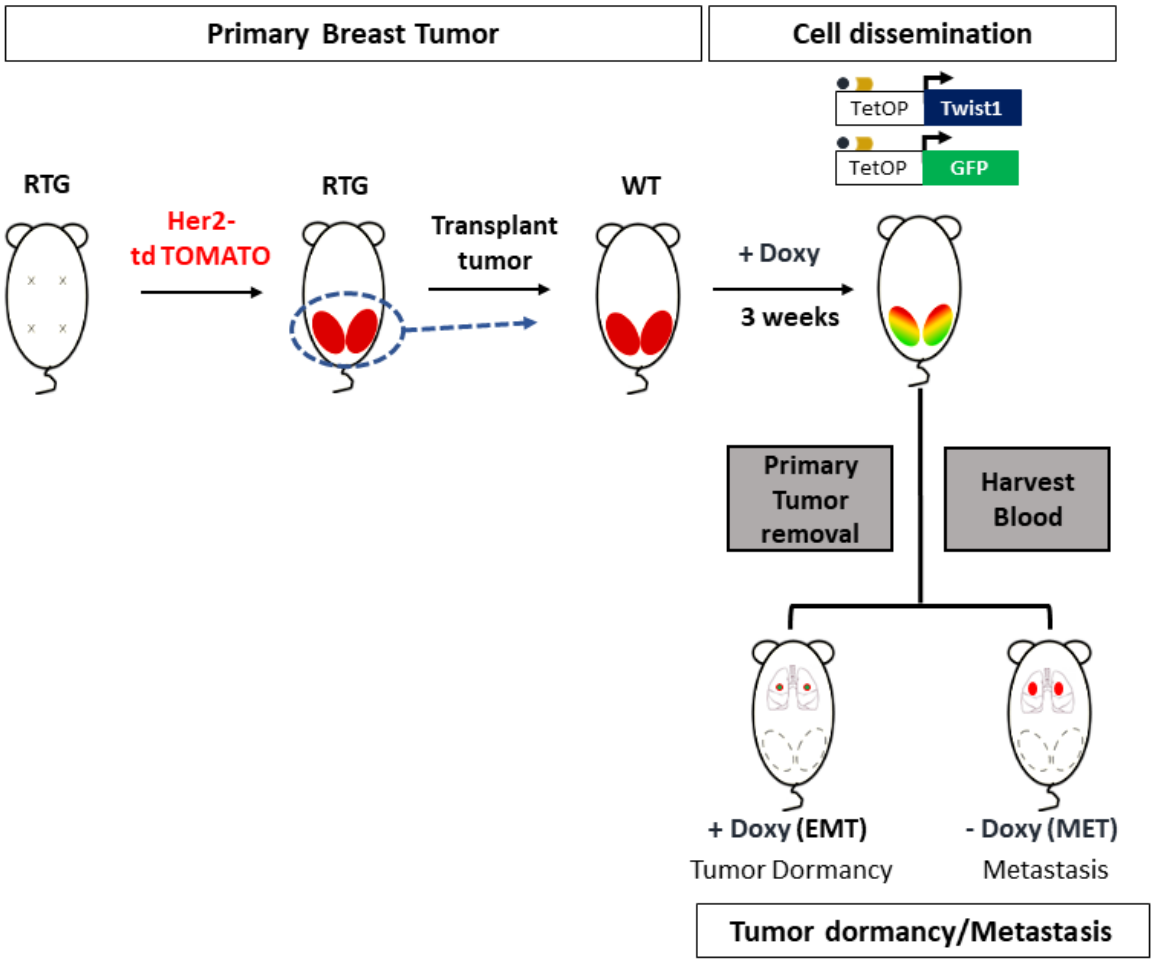


**Figure 2. Twist1-inducible Breast Cancer Tumor Dormancy Mouse Model**
Schematic of breast cancer mouse model. Generation of Her2-driven breast tumor by injection of Her2 lentivirus ($2x10^5$TU). Tumors that reached 1cm were transplanted from RTG to wild-type mice. 5mg/mL doxycycline was given for three weeks in drinking water. Mice were split into tumor dormancy group (continuous doxy) and metastasis group (stop doxy) after tumor removal.

**Doxycycline Induces GFP and Twist1 Expression in the Mammary Gland**

The RTG mice have both Twist1 and GFP controlled by the TetON promoter. To confirm that GFP can serve as fluorescent tag for visualizing Twist1 expression under doxycycline, mice received doxycycline for two weeks and the mammary glands were harvested for analysis. Immunofluorescent staining of GFP (green) and Twist1 (red) confirmed that only under doxycycline condition, GFP and Twist1 were expressed in the same cell of the mammary duct (Figure 3).

**Characterization of Her2-tdTomato Lentivirus Titer**

Prior to generating primary mammary tumors, the Her2-tdTomato lentivirus was validated for the expression of both Her2 and tdTomato fluorescence. The viral was also determined to ensure generation of primary tumors. The Her2-tdTomato lentivirus were transfected to HEK293T cells and the confocal image confirmed that the cells were positive for tdTomato fluorescence (Figure 4A). To determine that the infected cells express Her2 and tdTomato fluorescence, western blot and flow cytometry were performed. The western blot detected presence of Her2 protein expression (Figure 4B) and flow cytometry detected 25.4% of tdTomato positive cells (Figure 4C).

**Validation of Intraductal Injection Method to Generate Her2-driven Breast Tumors**

After the Her2-tdTomato lentivirus was validated and ready for injection, the method of delivery for the generation of mammary tumors also need to be validated. Female mice have five pairs of mammary glands. The Her2-tdTomato lentivirus was injected into the fourth pair of mammary ducts to generate mammary tumors (Figure 5A). Intraductal injections with trypan

blue dye demonstrated that intraductal injection of virus would spread throughout the entire ductal structure without any leaks (Figure 5B).

Four weeks after intraductal injection with Her2-tdTomato, primary mammary tumors formed. As depicted the mammary tumors are restricted within the mammary glands and not on the skin or other organs (Figure 6A). Hematoxylin & Eosin (H&E) histology showed characteristics of an invasive ductal carcinoma induced by Her2-tdTomato lentivirus (Figure 6B). Towards the bottom of the histology image is the normal ductal area that still maintained ductal structures. However, the top of the histology shows the invasive tumor area where normal ductal structures no longer exist and tumor cells present larger nuclei. Overall, these results suggest that intraductal injection is a good method for delivering Her2-tdTomato lentivirus to generate mammary tumors.

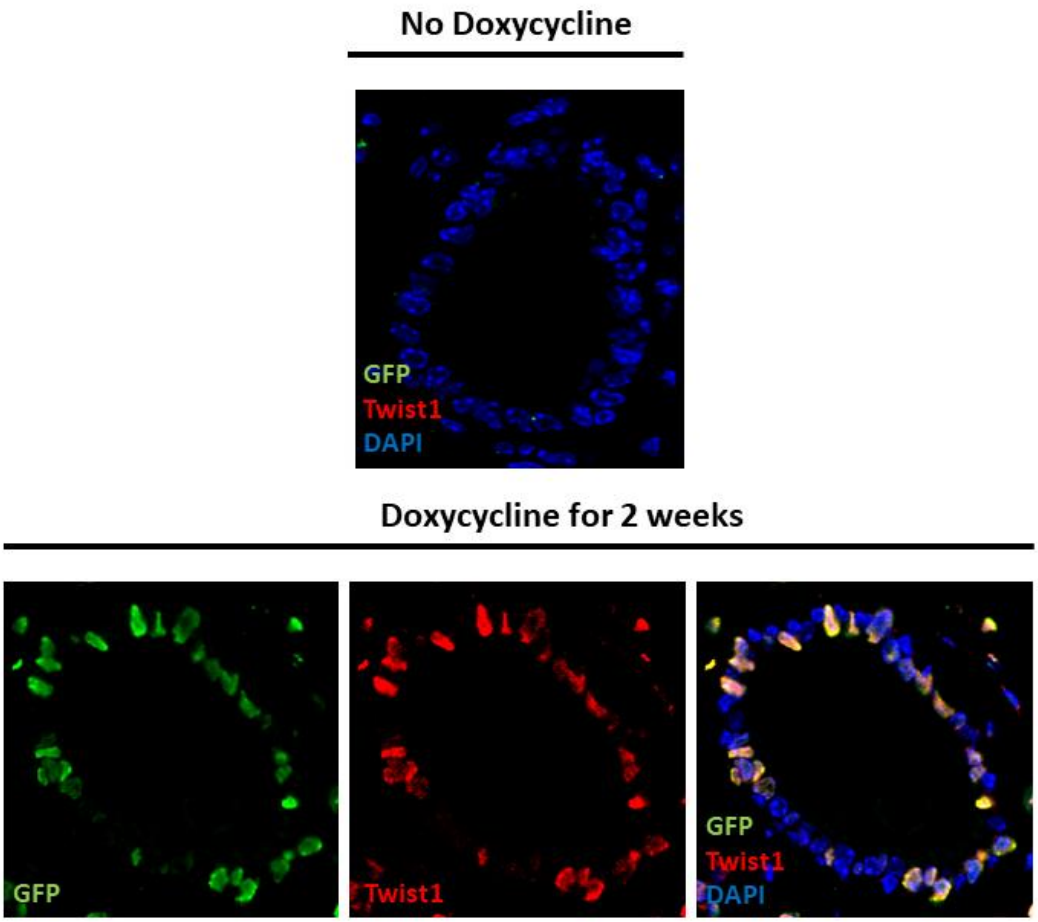


**Figure 3. Doxycycline Induces GFP and Twist1 Expression in the Mammary Duct**
Representative image of mammary glands stained with GFP (green), Twist (red), and DAPI (blue). Mice were treated with 5mg/mL of doxy for 2 weeks.

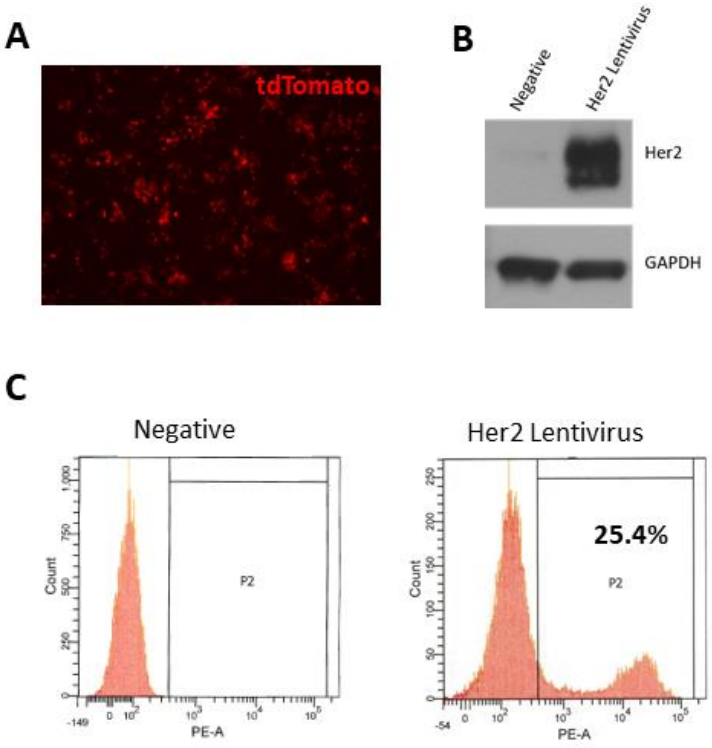


**Figure 4. Test Her2-tdTomato Lentivirus Titer**

(A) Representative Image of tdTomato positive signal (red) after infection.

(B) Western blot of Her2 protein expression

(C) FACS analysis of the percentage of tdTomato positive cells in the population. Control cells were measured to determine level of background. 25.4% are tdTomato positive cells.

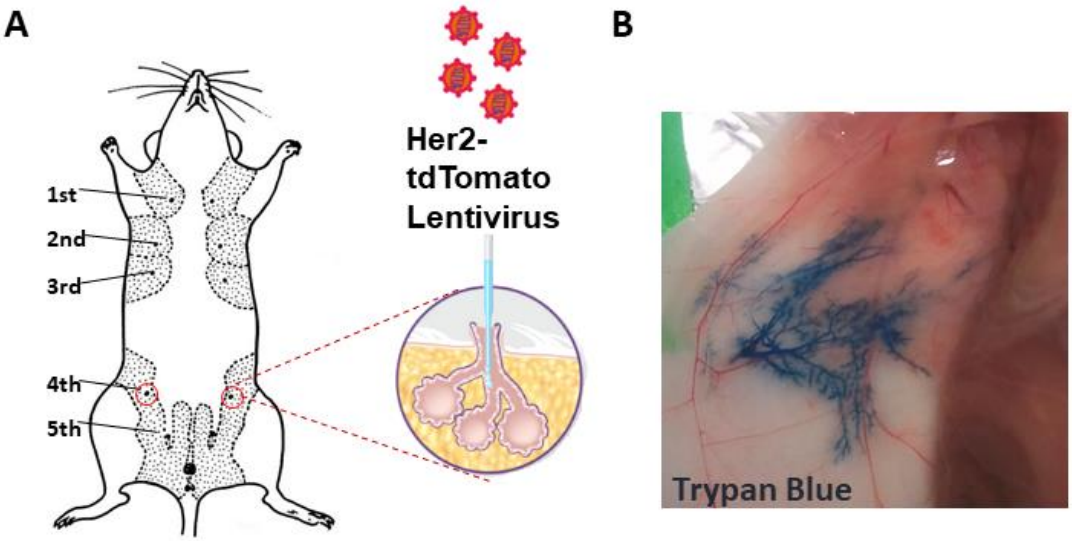


**Figure 5. Generation of Her2-driven Breast Tumors**

(A) Intraductal injection of Her2-tdTomato Lentivirus into the 4$^{th}$ mammary gland

(B) Representative image of injection of trypan blue dye into mammary gland to depict successful intraductal injection

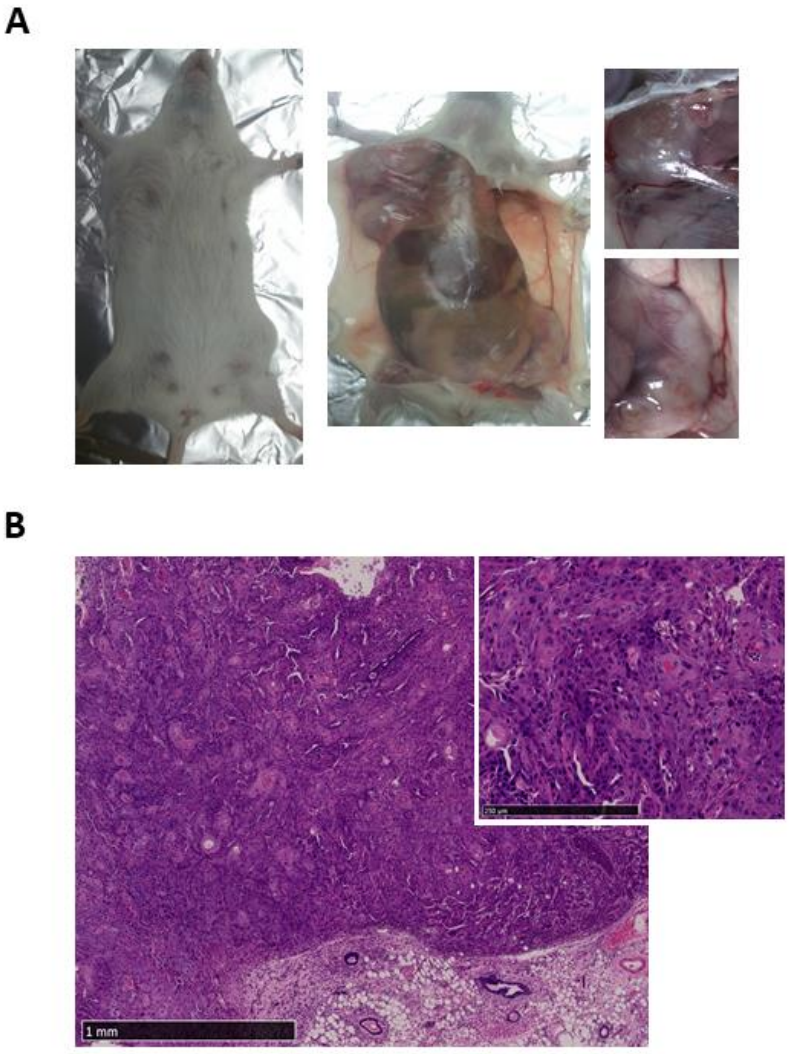


**Figure 6. Invasive Ductal Carcinoma Induced by Her2-tdTomato Lentivirus**

(A) Representative Image of Her2-driven breast tumor development 4 weeks post intraductal injections.

(B) Hematoxylin and Eosin Histology of Her2-driven breast tumor development.

**Doxycycline effectively controls Twist1 expression in Her2-driven Breast Tumors**

To confirm that the primary mammary tumors generated by the lentivirus were expressing Her2-tdTomato and doxycycline can induce GFP and Twist1, the mammary tumors were removed after three weeks of doxycycline induction. The mammary tumors were positive for both Her2 (red) and GFP (green), therefore suggesting doxycycline effectively induced Twist1 expression in Her2-driven breast tumors and can be visualized by GFP (Figure 7A). Her2 and Twist1 expression were further confirmed with immunofluorescent (IF) staining on mammary tumor paraffin sections. For no doxycycline condition, membranous Her2 signal were observed as expected in the Her2-driven breast tumor (Figure 7B). Under the doxycycline condition, both membranous Her2 and nuclear Twist1 expression were detected, therefore confirming doxycycline can control Twist1 expression in Her2-driven breast tumors.

Next, it was important to determine if Twist1 expression could induce Epithelial-Mesenchymal Transition (EMT) in the mammary tumors. Immunofluorescent staining and western blot were performed on primary mammary tumors for epithelial markers (E-cadherin) and mesenchymal markers (Fibronectin, Vimentin, N-cadherin). IF staining showed under the no doxycycline condition, the tumors primarily expressed epithelial marker E-cadherin (Figure 8A). In contrast, under doxycycline induction, the tumors underwent EMT as shown by decreased expression of epithelial marker E-cadherin and increased expression of mesenchymal marker Fibronectin. The western blot confirmed the IF staining results that once doxycycline was turned on in Her2 positive tumors, Twist1 was expressed and E-cadherin epithelial marker decreased while Vimentin and N-cadherin mesenchymal markers increased (Figure 8B).

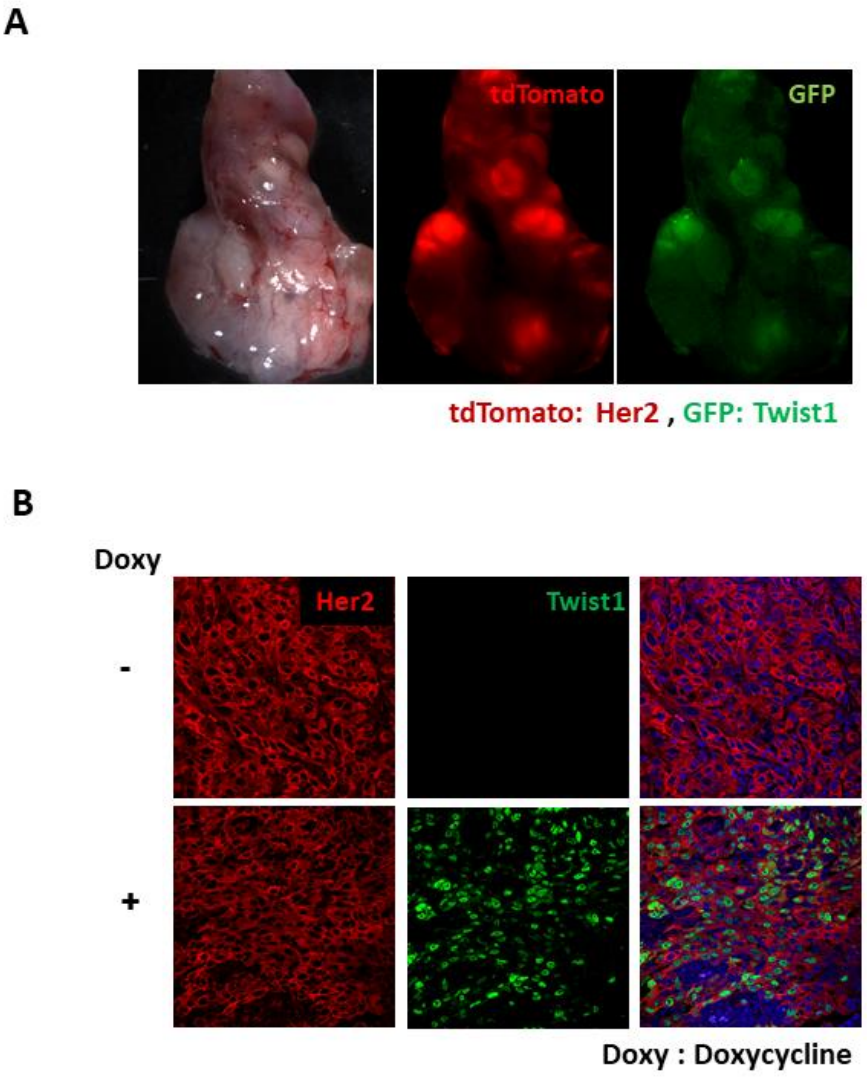


**Figure 7. Doxycycline Effectively Controls Twist1 Expression in Her2-Driven Breast Tumors**

(A) Representative image of Her2 primary breast tumor visualized under dissection scope. Mouse received 5mg/mL doxy for 3 weeks before tumor resection.

(B) Immunofluorescence staining with Her2 (red) and Twist1(green) on primary breast tumors embedded in paraffin and sectioned at 5 μm. Mice received 5mg/mL doxy for 3 weeks before tumor resection.

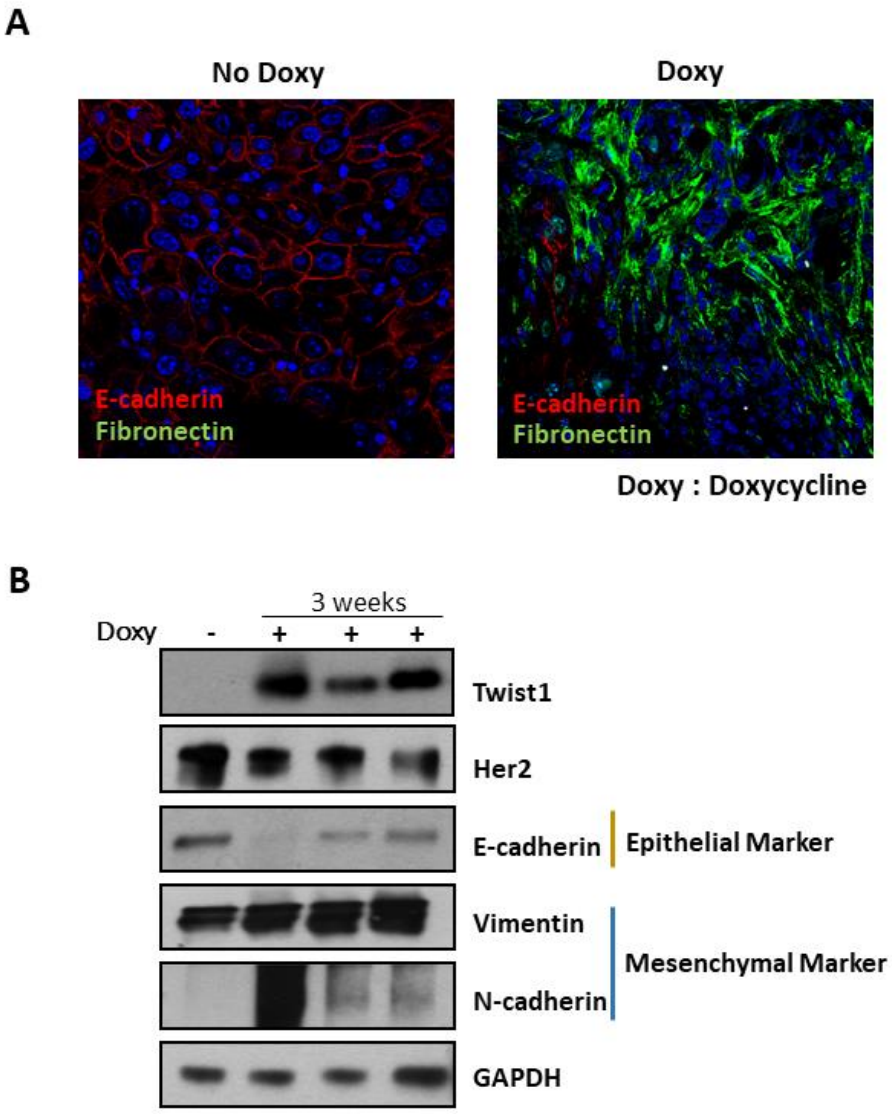


**Figure 8. Twist1 Induces EMT in Her2-driven Breast Tumors.**

(A) Representative Image of immunofluorescence staining of E-cadherin and Fibronectin on primary breast tumors embedded in paraffin and sectioned at 5 μm. 5mg/mL Doxy was given in drinking water to mice for 3 weeks before tumor resection.

(B) Western Blot of Her2, Twist1, and EMT Markers for mice without and with 5mg/mL doxycycline for three weeks. GAPDH is the loading control.

**Twist1 Expression Promotes Increase in Circulating Tumor Cells (CTCs) in the Blood**

Peripheral blood samples were harvested during the time course of 0, 4, 6, 9, 12, and 17 days doxycycline induction to analyze the presence of CTCs. Presence of CTCs were identified in the blood as tdTomato positive (red), Pan-cytokeratin positive (green), and CD45 negative (yellow) (Figure 9A). There were higher numbers of CTCs in the blood during the earlier treatment times of doxycycline (Figure 9B). This suggests that most tumor cell dissemination occurs early upon Twist1 induction.

**Twist1 Expression Promotes Tumor Cell Dissemination to the Lung**

To analyze whether tumor cells that disseminated into the circulation could travel and establish themselves in the lung, a time course of Twist1 induction by doxycycline for 2, 4, 6, 9, 12, 13, 17, and 20 days was performed. There were five mice for each time point of doxycycline induction. Twelve mice with Her2-tdTomato mammary tumors that did not receive doxycycline served as controls. The lungs were harvested after each time point and presence of disseminated tumor cells were identified by GFP positive cells (Figure 10A). On average, after only 2 days of doxycycline induction, there were 30 disseminated tumor cells found at the lung while the no doxycycline group had no disseminated tumor cells at the lung (Figure 10B). As the Twist1 induction time increased from day 4 to day 20, the number of disseminated tumor cells at the lung decreased to about 10-20 (Figure 10B). Since only five mice were used for each time point, the number of disseminated tumor cells is variable. But the data trend suggests that Twist1 expression is needed to promote tumor cell dissemination into circulation and distant organs. Furthermore, dissemination of tumor cells is strongest during earlier Twist1 induction time.

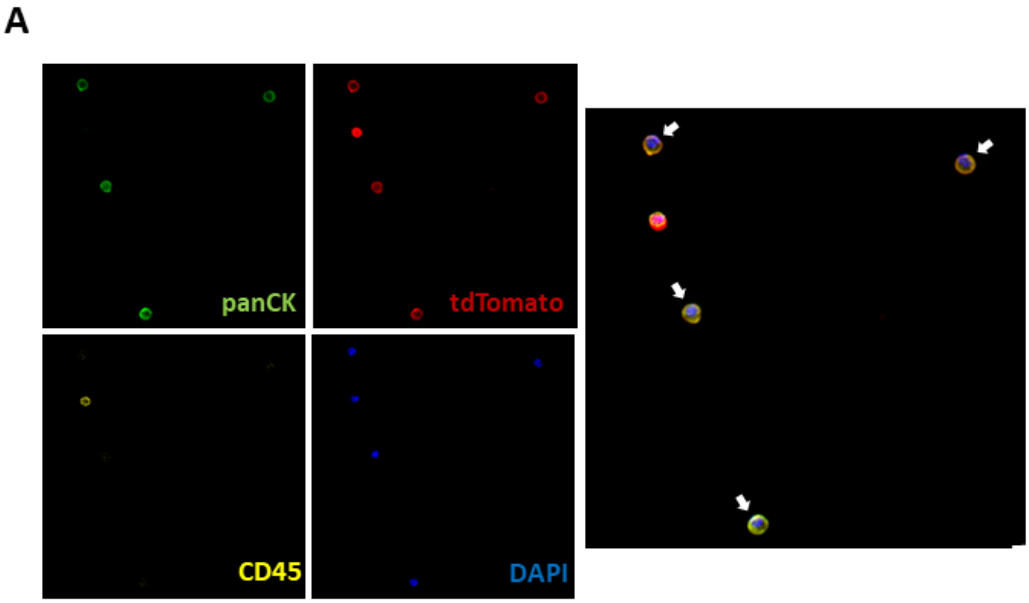


**Figure 9. Twist1 Induction Increases the Number of Circulating Tumor Cells (CTCs)**

(A) Representative immunofluorescent images of pan-cytokeratin (green), tdTomato (red), CD45 (yellow), and DAPI (blue). CTC positive cells are CD45- and CK+ (white arrows).

(B) Histogram represents number of circulating tumor cells (CTCs) for varying days of doxy treatment. The number of CTCs for each time point is represented above each bar.

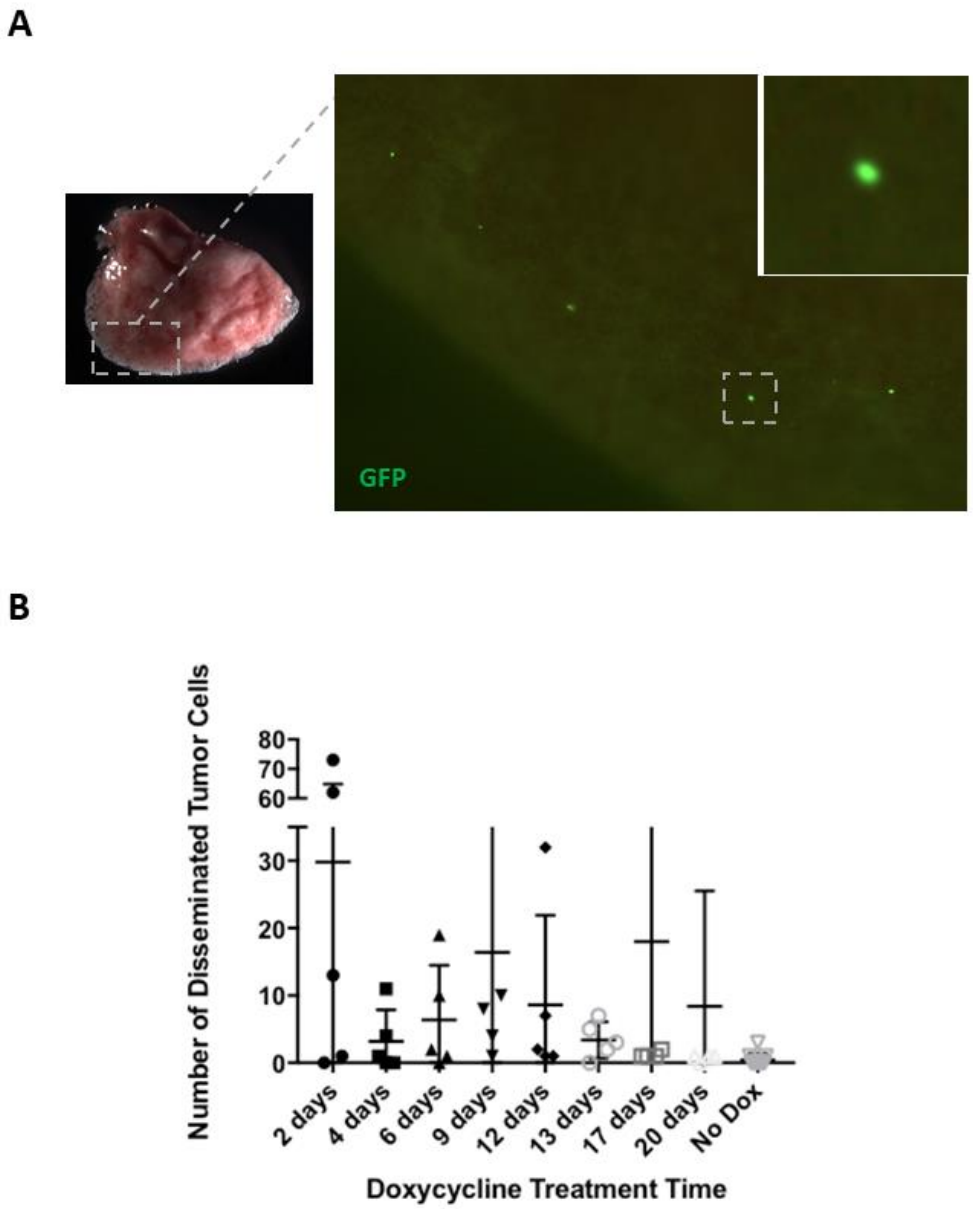


**Figure 10. Twist1 Promotes Tumor Cell Dissemination to the Lung**

(A) Representative Image of Lungs visualized under dissection scope. Disseminated tumor cells identified by single nuclear GFP signals. Mice were administered 5mg/mL doxy for 1 week.

(B) Scatter plot of the number of disseminated tumor cells (DTCs) for varying days of doxy treatment. Each dot represents one mouse and bar represents mean of each group. For Doxy treatment n=5 for each time point. For No doxy control, N=12.

**Twist1 Maintains Tumor Dormancy in Disseminated Tumor Cells at the Lung while**

After the mice received doxycycline for three weeks, primary mammary tumors were removed and the mice were split into two groups. The first group will receive doxycycline to maintain Twist1 expression and EMT. The second group will be withdrawn from doxycycline to stop Twist1 expression and induce MET. After 2-3 months, the lungs were harvested and sectioned for characterization by IF staining.

Under continuous doxycycline condition, the presence of disseminated dormant tumor cells were identified as single nuclear GFP cells in the lung (Figure 11A). Endogenous fluorescent signals from the single nuclear cell were confirmed by IF staining to express GFP and Her2, therefore confirming these cells are indeed disseminated tumor cells that remained dormant upon continuous Twist1 induction by doxycycline (Figure 12A).

Under stop doxycycline condition, there were no GFP cells present in the lung, consistent with the fact that doxycycline was withdrawn. Instead, there were tdTomato signals that spanned across multiple cells present in the lung (Figure 11B). The tdTomato positive cells expressed Her2 (green) by IF staining and confirmed that these cells are tumor cells that underwent metastatic outgrowth when Twist1 was turned off via doxycycline withdrawal (Figure 12B).

To determine the proliferative state of the disseminated tumor cells under continuous doxycycline condition, the lung sections were stained with ki67 proliferation marker and cleaved-caspase 3 apoptotic marker. There were ki67 positive signals for normal lung cells, however the disseminated tumor cell labelled by GFP was not positive for ki67 signal (Figure 13A). This indicates that the disseminated tumor cells under continuous Twist1 expression are not proliferating. Furthermore, there were cleaved-caspase3 positive for nearby normal lung cells, however the disseminated tumor cell was not positive for cleaved-caspase3 (Figure 13B).

Therefore, the disseminated tumor cells are not going through apoptosis. From this result, the disseminated tumor cells are not proliferating and not apoptotic, which may suggest when Twist1 is expressed continuously, the disseminated tumor cells are in a dormant state.

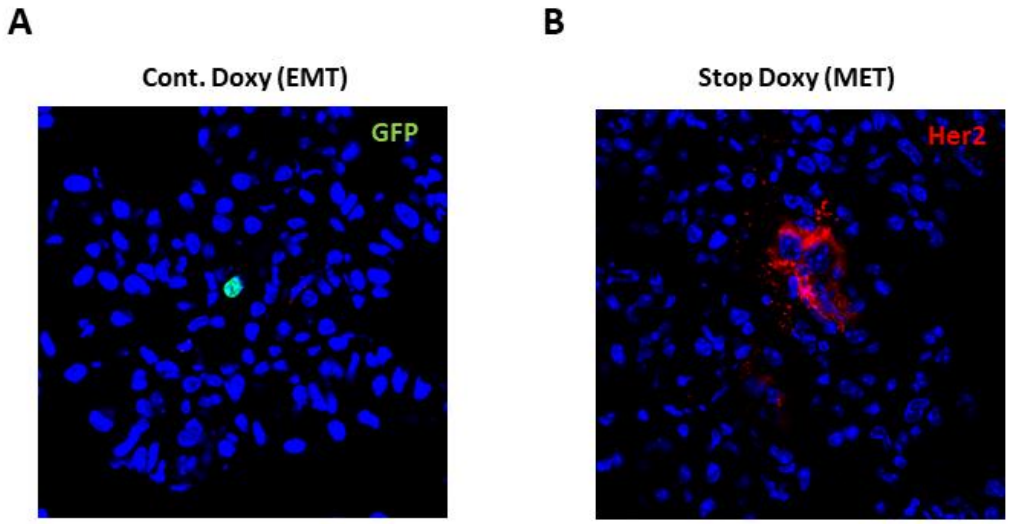


**Figure 11. Tumor Dormancy Observed When Twist1 is Turned On While Metastatic Outgrowth Observed When Twist1 is Turned Off.**

(A) Representative images taken by confocal microscope. Endogenous GFP signal (green) on frozen section of lung from mice with continuous doxy treatment for 50 days.

(B) Endogenous Her2 signal (red) on frozen section of lung from mice withdrawn from doxy for 50 days.

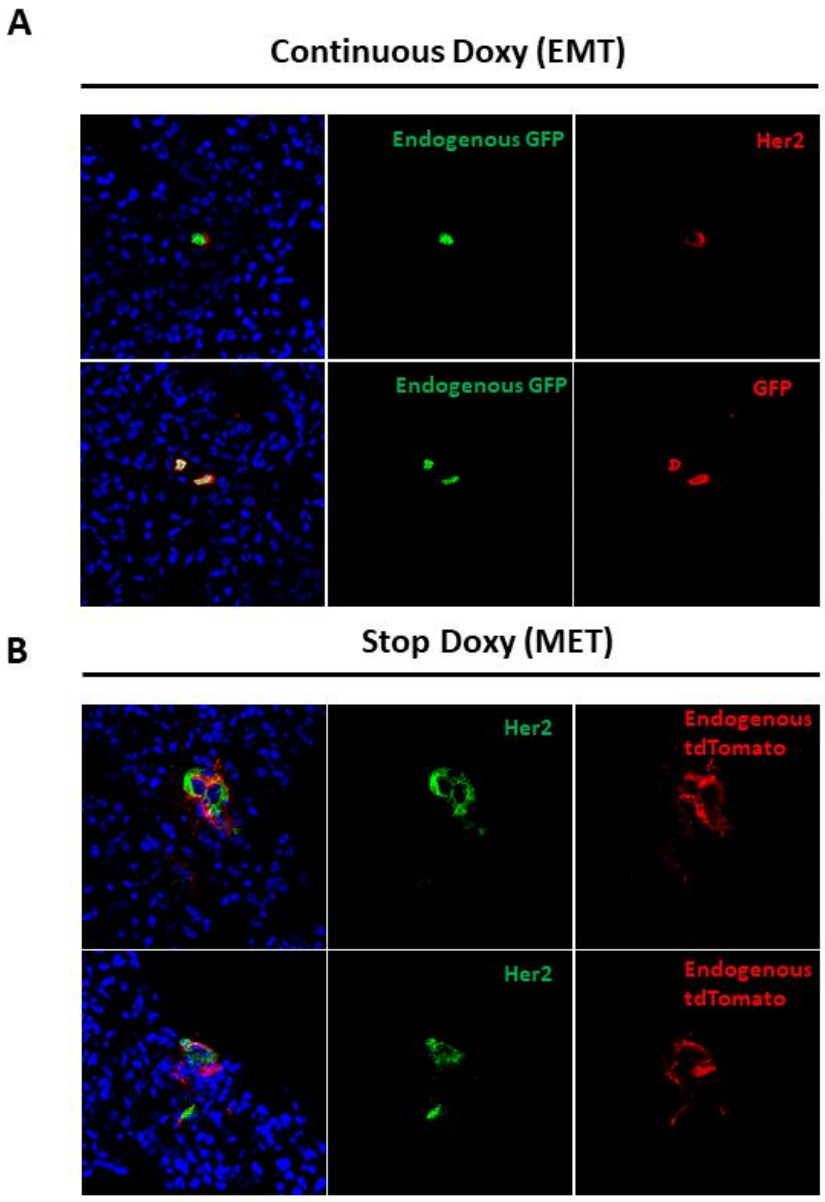


**Figure 12. Presence of Dormant Tumor Cells and Metastatic Growth at the Lung Identified by Immunofluorescent Staining**

(A) Immunofluorescent staining of frozen lung sections stained with Her2 and GFP to characterize dormant tumor cells under the continuous doxy condition.

(B) Immunofluorescent staining of frozen lung sections stained with Her2 to characterize metastatic growth under the stop doxy condition.

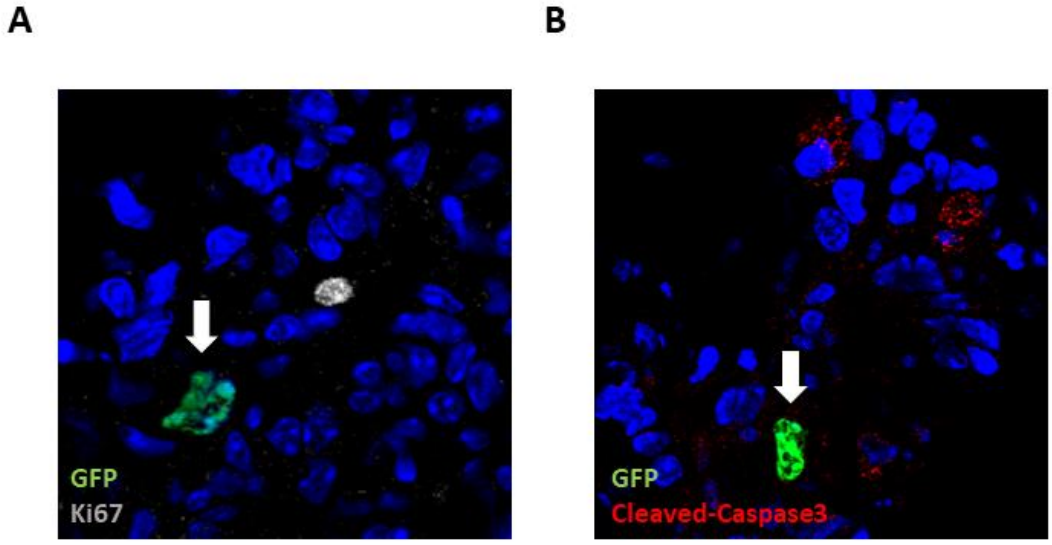


**Figure 13. Twist1 Maintains Tumor Dormancy in Disseminated Tumor Cells at the Lung**

(A) Immunofluorescent Staining of frozen lung sections stained with GFP and Ki67 proliferation marker. Mice received continuous 5mg/mL doxycycline for 50 days.

(B) Immunofluorescent Staining of frozen lung sections stained with GFP and Cleaved-Caspase3 apoptotic marker. Mice received continuous 5mg/mL doxycycline for 50 days.

**Reversion of EMT Promotes Outgrowth of Macrometastases in the Lung**

Next, we aim to see if disseminated tumor cells could establish macrometastases in distant organs. Under continuous doxycycline condition, 0 out of 12 mice (0%) formed macrometastasis (Figure 14A). In contrast, under stop doxycycline condition, 13 out of 19 mice (68%) formed macrometastasis with an average of 2 macrometastasis per lung (Figure 14A and B). These data suggest that the EMT program contributes to the dormant state of the disseminated tumor cells while reversing EMT is important to initiate metastatic outgrowth in distant sites; which was consistent with previous results from Tsai et al., 2012.

Furthermore, initially there were higher number of DTCs that reached the lung during Twist1 induction (Figure 10B). However, after 2-3 months the number of resulting macrometastasis is much smaller compared to the number of DTCs (Figure 14B and 10B), suggesting that only a very small percentage of DTCs can survive and form macrometastasis in the lung microenvironment.

The results, in part, has unpublished material from my co-authors Haeyun Jung and Kay Yeung. The thesis author was the primary investigator and author of this material.

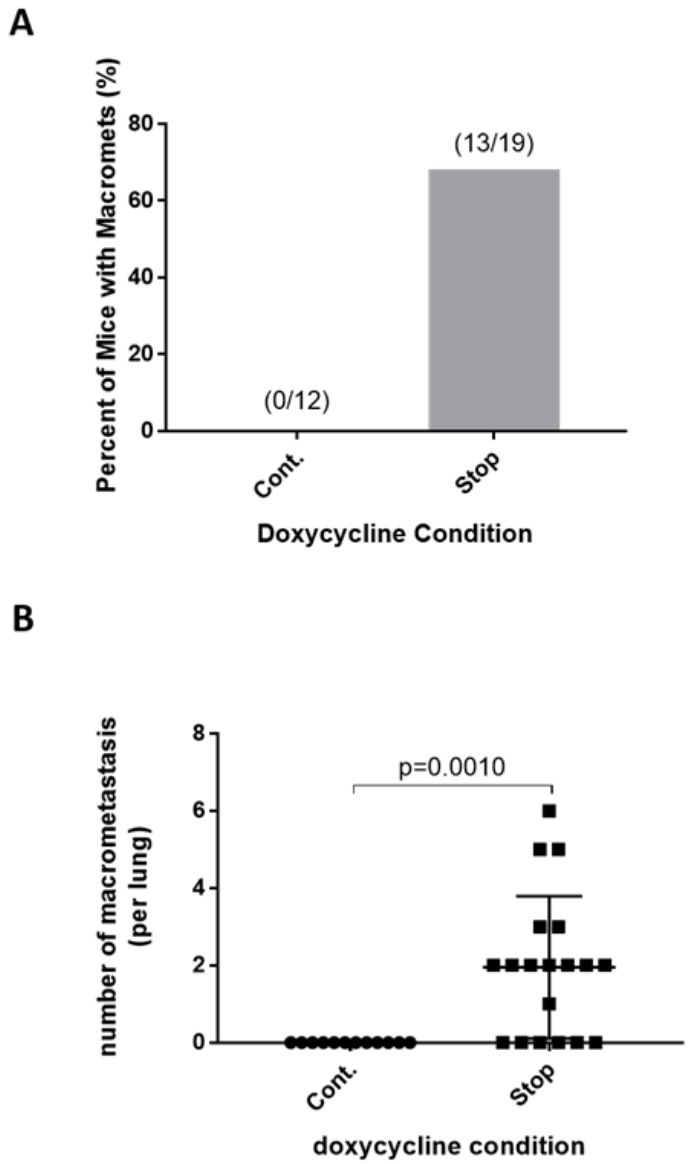


**Figure 14. Reversion of EMT Promotes Outgrowth of Macrometastasis at the Lung**

(A) Scatter plot of the number of macrometastasis per lung in continuous and stop doxy condition. Each dot represents one mouse and bar represents mean of each group. Comparisons by unpaired T-test for cont. and stop condition, P=0.0010.

(B) Histogram represents percent of mice with macrometastasis for continuous and stop doxy condition. The fraction of mice developing macrometastasis is represented above each bar.

# DISCUSSION

Approximately 90% of breast-cancer related deaths are due to distant tumor metastasis (Fidler, 2003). During metastasis, primary epithelial tumor cells undergo epithelial-mesenchymal transition (EMT) to gain the ability to disseminate from primary tumor, invade into systemic circulation, and migrate to a secondary site. Tumor cells that arrive in a secondary site can go into tumor dormancy for years until the microenvironment is suitable to reactivate proliferation by mesenchymal-epithelial transition (MET) to form macrometastases.

Studies have shown that transcription factor Twist1 is involved in inducing EMT and promoting tumor metastasis, however the mechanisms of metastasis remain unclear. One major obstacle in the study of metastasis is the lack of mouse model that can track tumor cells in a controlled and reproducible fashion. This study showed that the Twist1-inducible mouse model can utilize tdTomato and GFP fluorescence to track tumor cells throughout metastasis, therefore this model will serve as a useful tool in understanding metastasis.

tdTomato fluorescence effectively labelled for Her2-driven breast tumors and metastatic lesions while GFP directly labelled for Twist1 to visualize tumor cells throughout distinct stages of metastasis. Previous studies have shown that Twist1 expression is important for the initiation of EMT (Yang et al., 2008). This model was consistent with previous data that doxycycline can effectively control induction of Twist1 expression which then activated the EMT program in Her2-driven breast tumors. Furthermore, Twist1 expression promoted an increase of circulating tumor cells (CTCs) and presence of disseminated tumor cells (DTCs) in the lung. Interestingly, there were higher number of CTCs and DTCs during the earlier Twist1 induction time, suggesting most circulation and establishment at secondary site occur early upon Twist1 expression.

Although Twist1 is important for inducing EMT to initiate dissemination of tumor cells, previous studies have also shown that disseminated tumor cells undergo MET to form macrometastases when Twist1 expression is turned off (Tsai et al., 2012). After tumor removal, mice were split into two groups that either continued doxycycline treatment or stopped doxycycline treatment for 2-3 months. Consistent with previous results, our model demonstrated that continuous twist1 expression by doxycycline treatment maintained dormany in disseminated tumor cells at the lung. However, when Twist1 expression was turned off to initiate the reversal of EMT (MET), tumor cells regained proliferation and formed macrometastases at the lung. Previously there have been discussions about treatments aimed at targeting and turning off Twist1 and the EMT program. However, the crucial results found here indicate that turning off Twist1 may instead elicit a more negative effect and produce more macrometastases. Therefore, our mouse model can provide a better understanding of the mechanisms that contribute to metastasis and provide novel therapeutic targets to eradicate dormant metastatic tumor cells.

Based on our results, there were high numbers of CTCs following twist1 induction. Once the tumor cells go through circulation, the number of DTCs that reached the lung decreased. The number of macrometastases that developed in the lung after 2-3 months further decreased compared to the initial CTC and DTC count. This may suggest that once tumor cells reach the lung, the majority are unable to seed and proliferate in the new lung microenvironment. There may also be specific factors or changes in the microenvironment that is suitable for tumor cells to establish, remain dormant, and eventually proliferate into macrometastases. It is therefore important to understand what characteristics and gene signatures exist for the DTCs and lung microenvironment to determine what factors select tumor cells to establish at the lung and allow tumor cells to exit dormancy to form macrometastases.

Immunofluorescence staining with EMT markers, ki67 proliferation marker, and cleaved caspase-3 apoptotic marker can be used to characterize the state of cell proliferation of the DTCs that successfully established at the lung. This will determine whether DTCs have a more epithelial or mesenchymal phenotype and determine whether DTCs enter dormancy after establishing in the lung.

Furthermore, to determine the genetic signatures important for dormancy and metastasis, tumor cells from primary tumors, CTCs, and DTCs will be isolated through fluorescence-activated cell sorting (FACS) and submitted for RNA sequencing. The molecular profile of tumor cells from each metastatic state will be compared to determine any key factors that contribute towards tumor dormancy and metastasis.

Many studies have also mentioned that dormant tumor cells contribute towards chemoresistance due to the non-proliferative state of these cells (Gao et al., 2017). Therefore, it would be interesting to use this mouse model to determine whether dormant tumor cells can confer chemoresistance and whether reversing EMT along with chemotherapy treatment can effectively eradicate metastatic outgrowth. To do so, the mice will be split into four groups following tumor resection. First group will receive continuous doxycycline (EMT), second group will receive doxycycline with chemotherapy treatment (EMT + chemotherapy), third group will be withdrawn from doxycycline (MET), and last group will be withdrawn from doxycycline and receive chemotherapy treatment (MET + chemotherapy). If EMT contributes to chemoresistance in dormant tumor cells, it is expected that the MET + chemotherapy group would have decreased macrometastases compared with MET group. For EMT groups, it is expected micrometastases will be present but how chemotherapy affects EMT conditions is unknown.

# MATERIALS AND METHODS

## Generation of Twist1-inducible mice

All animal care and experiments were done in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. To generate a Twist1-inducible mouse model, I first crossed the ROSA-rtTA with TetOP-Twist1 mice to generate ROSA-rtTA:TetOP-Twist1 double transgenic mice. Then I crossed the double transgenic mice with TetOP-GFP mice to generate a ROSA-rtTA:TetOP-Twist1:TetOP-GFP triple transgenic mice (RTG). Since Twist1 and GFP are both controlled under the TetOP promoter in the presence of doxycycline, GFP will serve as fluorescent tag for Twist1 expression.

## Generation of Her2-tdTomato lentivirus and Her2-driven Breast Tumors

HEK293T cells were grown in 37°C, 5% $CO_2$ incubator, and maintained in culture medium containing DMEM, 10%FBS, and penicillin-streptomycin (50μg/mL). 24 hours before transfection, HEK293T cells were seeded in 150mm tissue culture dishes at a density of $6x10^6$ cells per dish. To generate Her2-lentivirus, HEK293T cells were transfected with envelope plasmid (pVSVG), package plasmid (Δ8.2), and viral vector pBOB-CAG-Neu-Tdt expressing Her2 labelled with tdTomato fluorescent protein using a plasmid DNA transfection TransIT-LT1 reagent (Mirus Bio). The transfection ratio of pVSVG: Δ8.2: pBOB-CAG-Neu-Tdt was at 1:9:10. The virus was harvested at 44 and 66 hour, filtered through 0.45μm filter (Millipore), mixed with sucrose containing buffer (10% sucrose in 1xPBS sterilized with 0.22μm filter) at 3:1 ratio, and centrifuged at 10,000g at 4°C for 4 hours. After centrifugation, the supernatant was

removed and the pellet was dried for 5 minutes. The viral pellet was resuspended in DMEM and stored in -80°C.

The lentivirus viral titer was determined by flow cytometry. HEK293T cells were seeded in a 6-well plate at a density of $4x10^5$ cells per well. After 24 hours, the virus was thawed on ice and added to the cells in serial dilution from 10µl to 0.25µl. After 48 hours, the cells were washed in 1xPBS and harvested for flow cytometry to analyze percentage of red fluorescent cells. The following equation was used to determine lentiviral titer:

$$Lentiviral\ Titer(TU/mL) = \left(\frac{\%red\ fluorescent\ cells}{100}\right) \times \left(\frac{Number\ of\ cells\ at\ time\ of\ transduction}{Volume\ of\ virus\ inoculum\ added\ in\ mL}\right)$$

A western blot was performed to further confirm the presence of Her2 protein expression.

The Her2-driven breast tumor was generated by intraductal injection of Her2-tdTomato lentivirus ($2x10^5$TU) into the $4^{th}$ mammary gland.

**Circulating Tumor Cell Collection, Count, and Visualization**

Blood samples were collected from submandibular vein and pooled together in collection tubes pre-coated with EDTA to prevent blood coagulation. Equal volume of Dextran solution was added into the blood and incubated at room temperature for one hour. The supernatant was transferred to a new tube and ammonium based red blood cell lysis buffer was added. Magnetic beads conjugated with CD45 antibody (Mojosort Mouse CD45 Nanobeads, BioLegend) was used as a final separation step to remove the remaining CD45+ cells. 2% of the enriched circulating tumor cell (CTC) was dried on a glass slide, fixed with 4% paraformaldehyde for 5 minutes, and stained with DAPI containing mounting media (Vector Labs).

Presence of CTC cells were defined by red and green double positive fluorescent cells. A digital automated fluorescence microscope (Keyance) was used to count the field of live cells

stained by DAPI in a pre-set area of the slide that was randomly selected. In the pre-set field, the number of red and green positive cells were manually counted. The estimated CTC count was calculated based on the proportion of CTC cells to the total DAPI positive cells.

**Immunohistochemical Staining**

Immunofluorescence staining on CTCs with mouse anti-CD45 antibody (BD Biosciences), rabbit anti-Pan-cytokeratin antibody (Abcam), and rabbit anti-Neu Antibody (Santa Cruz Biotechnology) were further conducted to validate CTCs.

Harvested tumors and lungs were either paraffin-embedded or frozen in OCT compound at -80°C and sectioned at 5-μm. Tumors and lungs were stained with a mouse anti-Twist1 antibody (Santa Cruz Biotechnology), rabbit anti-Neu antibody (Santa Cruz Biotechnology), mouse anti-E-cadherin (BD Biosciences), and rabbit anti-fibronectin (Sigma).

Endogenous mouse antigen were blocked with Mouse on Mouse blocking agent (Vector labs). Alexafluor dyes (Invitrogen) conjugated to corresponding species were used as secondary antibody. with DAPI containing mounting media (Vector Labs) was used as nuclear stain.

**Protein Concentration Measurement**

Protein concentrations were determined with Lowry assay (Bio-Rad). To make working reagent, 20μL of reagent S was added to 1mL of reagent A. 100μL of working reagent and 800μL of reagent B were added into each protein sample and incubated for 15 minutes at room temperature. After incubation, 900μL sample mixture was added into a cuvette and protein concentration was measured with a nanospectormeter (Implen). For standard curve, five different Bovine Serum Albumin at 0, 1,3, 5, and 7mg/mL was used.

**Western Blot**

Proteins dissociated from primary mammary tumor were denatured at 95°C for 5 minutes in 50mM DTT and 5X loading sample buffer to ensure the protein is denatured. Samples were loaded into RunBlue 4-12% pre-cast SDS gels and ran at 100V for 40 minutes and then 120V until loading dye reaches the bottom of the gel. Proteins were transferred onto a nitrocellulose membrane and ran at 200V for 120m minutes. The membrane was blocked with 5% milk in TBST for 60 minutes at room temperature with agitation. Primary antibodies were diluted in 1% BSA in TBST and incubated with membrane at 4°C for overnight with agitation. After primary antibody incubation, the membrane was washed 3 times for 5 minutes each with TBS-T. The corresponding secondary HRP conjugated antibody were diluted in 5% milk in TBST for 60 minutes at room temperature. The membrane was then washed 3 times for 5 minutes each with TBST.

The membrane was visualized with Amersham ECL Select$^{TM}$ Western Blotting Detection Reagent kit (GE Healthcare) and exposed on HyBlot CL autradiography Film.
The primary antibodies used for western blot were Twist1 (Santa Cruz Biotechnology), Neu (Santa Cruz Biotechnology), E-cadherin (BD Biosciences), Vimentin (BD Biosciences), N-cadherin (BD Biosciences), and GAPDH (GeneTex).

**Imaging**

Images were taken with Olympus FV-1000 confocal microscope and keyance microscope. Images were processed with ImageJ software version 1.51k (National Institute of Health). Graphs and statistical analysis were done on GraphPad Prism 7.

# REFERENCES

American Cancer Society. Cancer Facts & Figures 2018. Atlanta:American Cancer Society; 2014

Barrallo-Gimeno, A. and Nieto, M.A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 132, 3151–3161.

Cristofanilli, M., Budd, G.T., Ellis, M.J., Stopeck, A., Matera, J., Miller, M.C., Reuben, J.M., Doyle, G.V., Allard, W.J., Terstappen, L.W., and Hayes, D.F. (2004). Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 351(8), 781-91.

Dasgupta, A., Lim, A.R., and Ghajar, C.M. (2017). Circulating and disseminated tumor cells: harbingers or initiators of metastasis? Mol Oncol. 11(1), 40-61.

Eckert, M.A., Lwin, T.M., Chang, A.T., Kim, J., Danis, E., Ohno-Machado, L., and Yang, J. (2011). Twist1-induced invadopodia formation promotes tumor metastasis. Cancer Cell 19, 372–386.

Fidler I.J., (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 3(6), 453-458.

Gao X.L., Zhang M., Tang Y.L., and Liang X.H. (2017). Cancer cell dormancy: mechanisms and implications of cancer recurrence and metastasis. Onco Targets Ther 10, 5219-5228.

Hay, E.D. (1995). An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) 154(1), 8-20.

Kopp, H.G., Placke, T., and Salih, H.R. (2009). Platelet-derived transforming growth factor-beta down-regulates NKG2D thereby inhibiting natural killer cell antitumor reactivity. Cancer Res. 69, 7775–7783.

Korpal, M., Lee, E. S., Hu, G. and Kang, Y. (2008). The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J. Biol Chem. 282(22), 14910-4.

Labelle, M., and Hynes, R.O. (2012). The initial hours of metastasis: the importance of cooperative host-tumor cell interactions during hematogenous dissemination. Cancer Discov. 2, 1091–1099.

Lambert, A.W., Pattabiraman, D.R. and Weinberg, R.A. (2017). Emerging biological principles of metastasis. Cell 168, 670–691.

Moussavi-Harami, S.F., Wisinski, K.B., and Beebe, D.J. (2014). Circulating tumor cells in metastatic breast cancer: a prognostic and predictive marker. J Patient Cent Res Rev. 1(2), 85-92.

Qin, Q., Xu, Y., He, T., Qin, C., and Xu, J. (2012). Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. Cell Res. 22(1), 90-106.

Santamaria, P.G., Moreno-Bueno, G., Portillo, F., and Cano, A. (2017). EMT: Present and future in clinical oncology. Mol Oncol. 11(7), 718-738.

Tsai J.H., Donaher J.L., Murphy D.A., Chau S., and Yang J. (2012). Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell 22(6), 725-736.

Weinberg, R.A. The Biology of Cancer. 2007.

Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117, 927–939.

Yang, J., and Weinberg, R.A. (2008). Epithelial-Mesenchymal Transition: At the crossroads of development of tumor metastasis. Developmental Cell 14(6), 818-829.

Yao, D., Dai, C., and Peng, S. (2011). Mechanism of the mesenchymal-epithelial transition and its relationship with metastatic tumor formation. Mol Cancer Res. 9(12), 1608-20.

Yap, A.S., Brieher, W.M., and Gumbiner, B.M. (1997). Molecular and functional analysis of cadherin-based adherens junctions. Annu. Rev. Cell Dev. Biol 13, 199-146.