

5-2011

Investigating the Effects of Silencing EphA2 in Metastatic Breast Cancer Cells

Stephanie Erzinger

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations



Part of the [Biology Commons](#), and the [Cancer Biology Commons](#)

Recommended Citation

Erzinger, Stephanie, "Investigating the Effects of Silencing EphA2 in Metastatic Breast Cancer Cells" (2011). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 116.

https://digitalcommons.library.tmc.edu/utgsbs_dissertations/116

This Thesis (MS) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digitalcommons@library.tmc.edu.

INVESTIGATING THE EFFECTS OF SILENCING EPHA2 IN
METASTATIC BREAST CANCER CELLS

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Stephanie Ann Erzinger, B.S.
Houston, TX

May 2011

Dedication

To my dad, who has instilled in me the belief that, with diligence and a positive attitude, everything is possible.

Acknowledgements

Firstly, I would like to express my abundant gratitude and appreciation to Dr. Price, whose support and guidance has been crucial to the development of my project and to my development as a student researcher.

I would like to thank my colleagues and friends, Galina Kiriakova and Hui Liu, who have given me so much to look forward to in both my professional and personal life. Their assistance to me has been endless.

I would like to express many thanks to my committee members for taking the time to listen to my progress and for giving me advice and guidance that has allowed my project to grow. Thank you: Drs. Joya Chandra, Gary Gallick, Menashe Bar Eli, and Anil Sood. A special thanks goes to Han Hee-Dong, Ph.D. from Dr. Sood's laboratory for his help with the liposomal and nanoparticle preparation for my *in vivo* studies and to Lily Huang from Dr. Bar Eli's laboratory for generating lentivirus for use in stable knockdown experiments.

I would like to thank MD Anderson's Department of Cancer Biology, specifically the immunohistochemistry laboratory, for the valuable resources and insight that I was provided with to help my project move forward. I'd like to thank the Smith Research Foundation and Norman Hackerman Advanced Research Program for their funding over the course of my studies. I'm also grateful and appreciative to have had support from the Graduate School of Biomedical Sciences, the members of the Cancer Biology Program, and the lovely people in the Smith Research Building, who I have had the pleasure of getting to know over the past couple of years.

A special thank you goes to my family and close friends, whose support has been invaluable. Dad, Mom, Al, Spencer, Steve, Scott, Tallent, Tyler, and Josh, thank you for all you have done for me.

Investigating the Effects of Silencing EphA2 in Metastatic Breast Cancer Cells

Publication No. _____

Stephanie Ann Erzinger, BS

Supervisory Professor: Janet E. Price, PhD

EphA2, also known as ECK (epithelial cell kinase), is a transmembrane receptor tyrosine kinase that is commonly over-expressed in cancers such as those of the prostate, colon, lung, and breast. For breast cancers, EphA2 overexpression is most prominent in the ER-negative subtype, and is associated with a higher rate of lung metastasis. Studies conducted to demonstrate the role of EphA2 in a non-cancerous environment have shown that it is very important in developmental processes, but not in normal adult tissues. These results make EphA2 a prospective therapeutic target since new therapies are needed for the more aggressive ER-negative breast cancers. A panel of breast cancer cell lines was screened for expression of EphA2 by immunoblotting. Several of the overexpressing cell lines, including BT549, MDA-MB-231, and HCC 1954 were selected for experiments utilizing siRNA for transient knockdown and shRNA for stable knockdown. Targeted knockdown of EphA2 was measured using RT-PCR and immunoblotting techniques.

Here, the functions of EphA2 in the process of metastasis have been elucidated using *in vitro* assays that indicate cancer cell metastatic potential and *in vivo* studies that

reveal the effect of EphA2 on mammary fat pad tumor growth, vessel formation, and the effect of using EphA2-targeting siRNA on pre-established mammary fat pad tumors. A decrease in EphA2 expression both *in vitro* and *in vivo* correlated with reduced migration and experimental metastasis of breast cancer cells. Current work is being done to investigate the mechanism behind EphA2's participation in some of these processes. These studies are important because they have contributed to understanding the role that EphA2 plays in the progression of breast cancers to a metastatic state.

Table of Contents

Approval Signatures	i
Title Page	ii
Dedication	iii
Acknowledgements	iv
Abstract	vi
Table of Contents	viii
List of Illustrations and Figures	xiii
List of Tables	xvi
List of Abbreviations	vii
Chapter 1: Introduction & Background	1
Introduction.....	2
Understanding the Anatomy of the Breast.....	2
Predisposition and Risk Factors.....	6
Types of Breast Cancer.....	6
Heterogeneity within Breast Cancers Creates Subtypes.....	8
Breast Cancer Staging.....	10
Treatment Strategies.....	12
Introduction to the Eph Subfamily and EphA2.....	14
EphrinA1 Ligand-EphA2 Receptor Interactions.....	17
The Role of EphA2 in Cancer.....	19
Regulation of EphA2.....	21

Estrogen and c-myc.....	21
Ras.....	22
E-Cadherin.....	23
p53.....	25
Low Molecular Weight Protein Tyrosine Phosphatase.....	26
Involvement of EphA2 in Metastatic Breast Cancer.....	28
Metastasis.....	28
EphA2 Involvement in Breast Cancer.....	29
Specific Aims of the Project.....	30
Chapter 2: Materials and Methods.....	32
Cell Lines.....	33
Cell Culture Conditions.....	33
Preparation of Whole Cell Lysates.....	34
Immunoblot Analysis.....	34
Isolation of RNA and Quantitative Real Time Polymerase Chain Reaction.....	35
siRNA Transfection.....	36
Migration Assay.....	37
ELISA for VEGF.....	37
Co-Immunoprecipitation Assay.....	38
Lentiviral Delivery of shRNA for Stable Silencing of EphA2.....	39
Agarose Colony Formation Assay.....	40
Immunocytochemistry for Fluorescence and Confocal Imaging.....	41
Animals.....	42

Mammary Fat Pad Tumor Growth.....	42
Experimental Metastasis.....	42
Liposomal and Chitosan Nanoparticle Treatment.....	43
Homogenization of Tumor Tissue for Protein Analysis.....	44
Immunohistochemistry for CD31 on Frozen Sections.....	44
Immunohistochemistry on Paraffin Embedded Tissue.	45
Densitometry.....	45
Statistical Analysis.....	46
Chapter 3: Specific Aim I – Utilization of Targeted shRNA to Study the Effects of	
Stable EphA2 Reduction <i>In vitro</i> and <i>In vivo</i>.....	47
Introduction.....	48
Results: <i>In vitro</i> Studies.....	50
Demonstration of Stable EphA2 Knockdown in MDA-MB-231 Luc,	
HCC1954, and BT549 Breast Cancer Cell Lines after Transduction with	
Lentivirus Containing EphA2-Targeting Constructs.....	50
MDA-MB-231 Luc and BT549 Cells Transduced with LV3 Show	
Reduced Migration Towards the Chemoattractant EGF Compared to the	
Control.....	53
MDA-MB-231 Luc cells with Stably Reduced EphA2 Levels Show No	
Change in Anchorage Independent Growth Capability.....	56
Results: <i>In Vivo</i> Studies.....	59
Mammary Fat Pad Tumor Growth was Reduced in Mice Injected with the	
Stable MDA-MB-231 Luc LV3 Cell Line.....	59

Tumor Protein Analysis Revealed Decreased EphA2 Protein Levels in the Mammary Fat Pad Tumors of Mice Injected with MDA-MB-231 Luc LV3 Cells.....	62
The MVD in MDA-MB-231 Luc LV3 Mammary Fat Pad Tumors was Reduced Compared to the MVD in MDA-MB-231 Luc LVNC Mammary Fat Pad Tumors.....	65
MDA-MB-231 Luc LV3 Cells Develop Fewer Experimental Lung Metastases in Comparison to LVNC Cells After Intravenous Injection into Mice.....	68
Discussion: Utilization of Targeted shRNA to Study the Effects of Stable EphA2 Reduction <i>In vitro</i> and <i>In vivo</i>	71
Chapter 4: Specific Aim II – siRNA-Mediated Transient Reduction of EphA2.....	77
Introduction.....	78
Results: <i>In vitro</i> Studies.....	80
Demonstration of EphA2 Expression at the Protein and mRNA Levels in a Variety of Breast Cancer Cell Lines.....	80
Use of EphA2-Targeting siRNA to Transiently Reduce Protein Levels in the MDA-MB-231, HCC1954, and BT549 Cell Lines.....	84
Cells with Transiently Reduced EphA2 Protein Levels Exhibited a Decrease in Migratory Ability Towards Chemoattractants.....	87
Investigation into the Relationship between EphA2 and EGFR in Promotion of Cancer Cell Migration.....	90

EphA2 and EGFR Co-localize at the Plasma Membrane of MDA-MB-231 Luc Cells.....	93
Immunoprecipitation of EphA2 from MDA-MB-231 Luc Cell Protein Lysates Did Not Pull Down of EGFR After Stimulation with EGF.....	96
Comparison of Vascular Endothelial Growth Factor Release in Cells Treated with Non-Targeting siRNA and EphA2-Targeting siRNA.....	99
Results: <i>In vivo</i> Studies.....	102
Delivery of EphA2-Targeting siRNA by DOPC Liposomes or Chitosan Nanoparticles had No Significant Impact on Tumor Growth Compared to the Corresponding Control.....	106
Analysis of Tumor Protein Lysate Revealed EphA2 Protein Levels Parallel the Trend Seen in Tumor Growth between the Various Experimental Groups.....	109
Discussion: Utilization of Targeted siRNA to Study the Effect of Transient EphA2 Reduction <i>In vitro</i> and <i>In vivo</i>	112
Chapter 5: Summary & Conclusions.....	121
Summary.....	122
Future Directions.....	130
Conclusions.....	132
References.....	133
Vitae.....	151

List of Illustrations and Figures

Chapter 1: Introduction and Background

Figure 1	Basic Breast Anatomy.....	4
Figure 2	Cellular Components of Breast Ducts and Lobules.....	5
Figure 3	Structure of Eph Receptors and Ephrin Ligands.....	16
Figure 4	Ras Regulation of EphA2 and Ephrin A1 Expression.....	24
Figure 5	Summary of EphA2 Regulation.....	27

Chapter 3: Specific Aim I – Utilize Targeted shRNA to Study the Effects of Stable EphA2 Reduction *In vitro* and *In vivo*

Results: *In vitro* Studies

Figure 6	Demonstration of EphA2 Knockdown in MDA-MB-231 Luc, HCC1954, and BT549 Cell Lines after Transduction with Lentivirus.....	52
Figure 7	Cells Transduced with LV3 Showed Attenuated Migration Towards EGF.....	55
Figure 8	Reduced Levels of EphA2 Do Not Affect Anchorage Independent Growth.....	58

Results: *In vivo* Studies

Figure 9	Mammary Fat Pad Tumors with Stably Reduced EphA2 Showed Decreased Tumor Growth.....	61
Figure 10	EphA2 Protein Levels Were Decreased in Mammary Fat Pad Tumors with Stably Reduced EphA2.....	64

Figure 11	Reduced MVD in MDA-MB-231 Luc LV3 Mammary Fat Pad Tumors Compared to MDA-MB-231 Luc LVNC.....	67
Figure 12	Breast Cancer Cells with Stably Reduced EphA2 Expression Do Not Develop Lung Metastases as Efficiently as Breast Cancer Cells Overexpressing EphA2.....	70

Chapter 4: Specific Aim II – Utilize Targeted siRNA to Study the Effect of Transient EphA2 Reduction *In vitro* and *In vivo*

Results: *In vitro* Studies

Figure 13	EphA2 Protein and mRNA Expression Levels.....	83
Figure 14	siRNA-Mediated Transient Reduction of EphA2 Protein Levels.....	86
Figure 15	Transient EphA2 Reduction Attenuates Migration Towards Chemoattractant.....	89
Figure 16	Transiently Reducing EphA2 Levels Leads to a Reduction in EGFR Expression.....	92
Figure 17	Demonstration of EphA2 and EGFR Co-localization.....	95
Figure 18	Immunoprecipitation of EphA2 Does Not Lead to EGFR Pull Down.....	98
Figure 19	VEGF Release in Metastatic Breast Cancer Cell Lines with Attenuated Levels of EphA2 Receptor Protein Levels.....	101

Results: *In vivo* Studies

Figure 20	Successful Delivery of Fluorescently Labeled DOPC Liposomes to MFP Tumors.....	105
Figure 21	Tumor Growth in siRNA Treated Animals.....	108

Figure 22	EphA2 and EGFR Protein Levels in Animal Tumor Tissue Treated with PBS, DOPC Liposomes, and Chitosan Nanoparticles.....	111
-----------	---	-----

Discussion

Figure 23	Proposed Mechanism of EphA2's Role in Rac1 Activation and Migration.....	116
-----------	---	-----

List of Tables

Introduction

Table 1	Basic Breast Cancer TNM Staging.....	11
Table 2	Summary of Notable Characteristics of Primary Cell Lines Used in this Thesis Research.....	31

List of Abbreviations

AJCC	American Joint Committee on Cancer
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
c-cbl	Casitas B-lineage Lymphoma (mouse)
cDNA	Complementary Deoxyribonucleic Acid
DAB	Diaminobenzidine
DCIS	Ductal Carcinoma <i>in situ</i>
DMSO	Dimethylsulfoxide
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphatidylcholine
Eck	Epithelial Cell Kinase
ECM	Extra Cellular Matrix
EGF	Edpidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
Ephrin	<u>Eph</u> <u>R</u> eceptor <u>I</u> nteracting (ligand)
ER	Estrogen Receptor
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GPI	Glycophosphatidylinositol
GRB2	Growth Factor Receptor Bound Protein 2

HER2/neu	Human Epidermal Growth Factor Receptor 2
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
i.p.	Intraperitoneal Injection
i.v.	Intravenous Injection
IVIS	In Vitro Imaging System
kDa	kiloDalton
LCIS	Lobular Carcinoma <i>in situ</i>
mRNA	Messenger Ribonucleic Acid
MFP	Mammary Fat Pad
MMP	Matrix Metalloproteinase
MVD	Microvascular Density
NaCl	Sodium Chloride
Na ₃ VO ₄	Sodium Orthovanadate
NGS	Normal Goat Serum
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-Kinase
PR	Progesterone Receptor
RISC	RNA-Induced Silencing Complex
RNAi	RNA Interference Pathway
ROI	Region of Interest
RTK	Receptor Tyrosine Kinase

SDS-PAGE	Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis
SHC	Src-Homology Domain-Containing Protein
SHP2	SH2 Domain-Containing Tyrosine Phosphatase 2
siRNA	short interfering Ribonucleic Acid
SLAP	Src-Like Adaptor Protein
TBST	Tris-Buffered Saline Tween-20
TNE	Tris, NaCl, EDTA Buffer
TNM	Tumor-Node-Metastasis
TPP	Thiamine Pyrophosphate
VEGF	Vascular Endothelial Growth Factor

**- Chapter 1 –
Introduction & Background**

Introduction

Cancer has continuously been a significant source of health concern in countries all over the world (1). It is the second leading cause of death in the United States, lagging only behind heart disease (1). While there are many types of cancer, breast cancer is one of particular interest due to the fact that there will be an estimated 209,060 new cases and 40,230 deaths attributed to this disease in 2010 alone (1). In women, it is the most commonly diagnosed cancer and is the second leading cause of cancer-related deaths (1). Furthermore, the disparity between the number of new diagnoses and annual deaths corroborate established findings that breast cancers are considerably variable and may or may not lead to lethality (2). Disease dissemination to distant sites within the body, a process called metastasis, is the major contributor to high breast cancer mortality rates (3). Overcoming the limitations in our understanding of metastasis and breast cancer biology requires further research and investigation so that new treatments may be developed and current therapies may be improved (2).

Understanding the Anatomy of the Breast

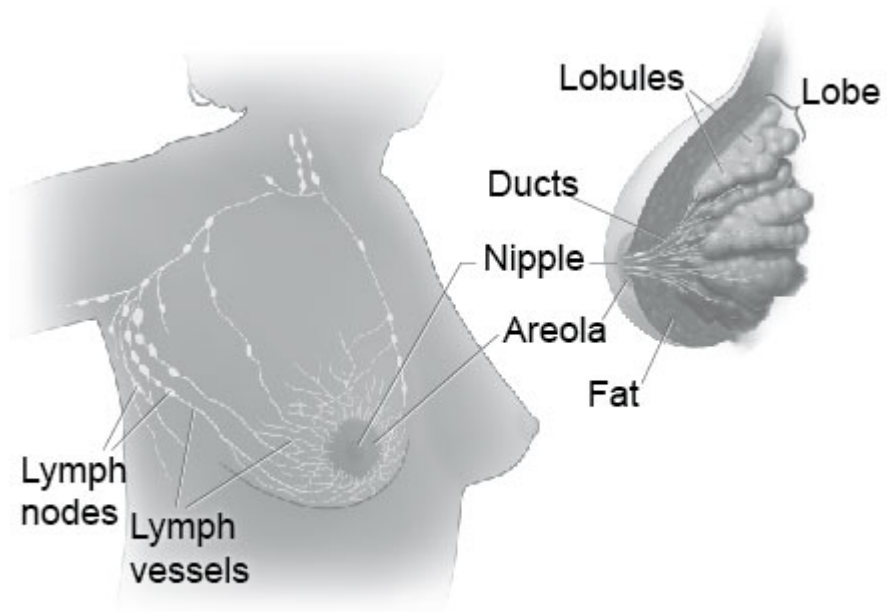
It is important to begin with a foundation in basic breast anatomy and function in order to understand the role that these anatomical features and processes play in the development and progression of breast cancers.

Mammary glands are a unique feature of mammals that function to secrete milk as a means of providing nourishment and transferring passive immunity to newborn organisms (4). In humans, the mature female breast consists of approximately 15-20 lobes that radiate outward from the nipple (4) (Figure 1). These lobes are composed of

smaller structures, called lobules (4). Acini are the hollow, sac-like constituents of the lobules that are responsible for producing and storing milk during pregnancy (4, 5). Tubular structures, called ducts, form a network of branched passages that initiate at the lobules and ultimately converge to provide a single route to the exterior through the nipple (4). The remainder of the breast is composed of adipose tissue and connective tissue infiltrated with lymph and blood vessels (5).

At the cellular level, both the lobules and the ducts are composed of an inner luminal epithelial cell layer and outer myoepithelial cell layer (6) (Figure 2). The myoepithelial cells, also called basal cells, are then followed by a lining of basement membrane (6, 7). The ductal myoepithelial cells differ from the acinar myoepithelial cells in that they are layered continuously around the luminal cells, whereas acinar myoepithelial cells are discontinuously layered (6). While there are many factors that play a role in characterizing breast cancers, the luminal or basal-like gene expression patterns seen in various tumor samples has led to the classification of subtypes of breast cancers and has helped to create a basic level of organization within this otherwise heterogeneous disease (8).

Figure 1
Basic Breast Anatomy

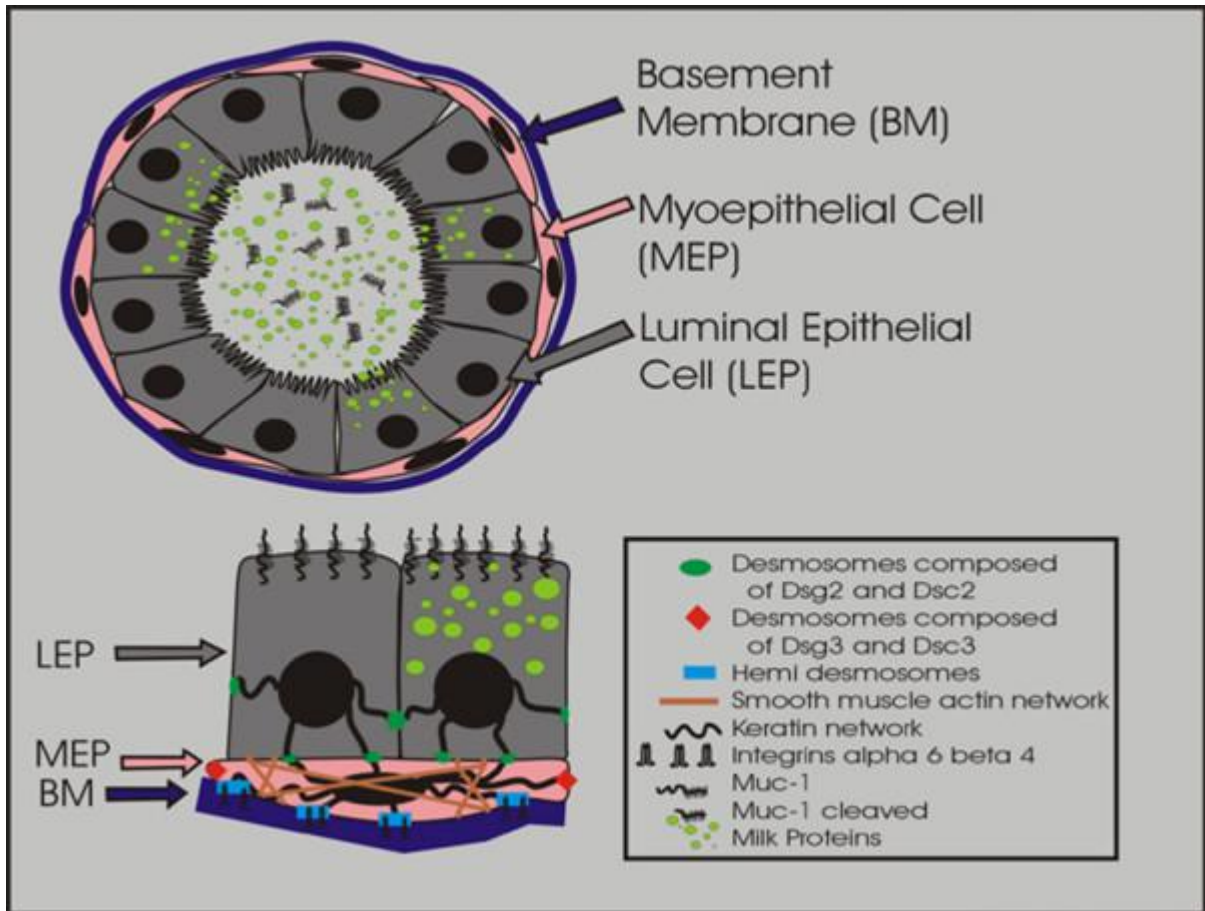


Reference (9)

Permission to Use Figure Granted by National Cancer Institute

Figure 2

Cellular Components of the Breast Ducts and Lobules



Breast Cancer Research

Reference (6)

Permission to Use Figure Granted by Original Publisher, BioMed Central

Predisposition and Risk Factors

Breast cancer may affect any person, but there are certain well-known factors that indicate a higher likeliness for disease development in select individuals (10). The most defining risk factors are age and sex (11). While men can be afflicted with this disease, the number of diagnoses and deaths in men are significantly fewer than in women and the majority of women diagnosed are normally 50 years of age or older (10, 11). Other factors that potentiate an increased risk include, but are not limited to, early onset of menstruation or late onset of menopause, obesity, becoming pregnant at an older age, familial history of breast cancer, and inheritance of genetic alterations (11). Mutations in either of the two genes, BRCA1 and BRCA2, are known for heightening the risk of breast cancer development and are found in 5-10% of patient cases (12). Furthermore, TP53 and PTEN are examples of two other genes that may harbor mutations and inheritance of these altered copies increases an individual's risk of disease development (13).

Types of Breast Cancer

As aforementioned, breast cancer is a diverse disease originating from a genetic predisposition or accumulation of spontaneous somatic mutations over time (18). However, not all breast disorders are considered to be cancerous and many non-cancerous conditions do not share the same projected risk of maturing into malignant disease (14).

Benign breast lesion is a term that collectively describes abnormalities of the breast that have varying risks for developing cancer, however, this risk may be higher or

lower depending on the specific condition (14). Examples of irregularities that are considered to have no associated risk of breast cancer development include lipomas, mastitis, and lymphadenitis (14). On the other hand, atypical ductal and lobular hyperplasia represent benign lesions that confer a higher potential to become malignant (14). Despite the disparity between these conditions and their associated cancer development risk, benign breast lesions are not contributors to breast cancer mortality rates and, if treated early, will not develop into a malignancy (14).

However, if left untreated, atypical ductal and lobular hyperplasia may lead to ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS), respectively (15). DCIS and LCIS are considered to be intermediary lesions that are precursors to invasive cancers (15, 16). DCIS is the more common form of *in situ* breast cancer and has a higher tendency to become invasive (15). Invasive forms of the disease have penetrated the basement membrane and infiltrated the surrounding tissue (15). Approximately 80% of breast cancer patients have invasive ductal carcinoma (IDC), whereas only 10% have invasive lobular carcinoma (ILC), also called infiltrating lobular carcinoma (15). Cancer cells constituting an invasive carcinoma have the potential to spread to regional lymph nodes and blood vessels where they will be disseminated throughout the body and could create tumors at distant sites (15).

A less commonly diagnosed type of breast cancer is called inflammatory breast cancer. Affecting less than 5% of all breast cancer patients, this form of the disease is not associated with the development of a lump in the breast (17). Rather, its symptoms include redness, swelling, and inflammation as a result of lymph vessel blockage by

cancer cells and is often misdiagnosed (17). This rare disease is aggressive, highly metastatic, and has a 5 year survival rate of less than 5% (17).

Heterogeneity within Breast Cancers Creates Subtypes

Transcriptional profile analysis and subsequent hierarchical clustering of data from a variety of breast tumor samples and breast cancer cells lines have revealed subtypes with specific genetic expression patterns (8, 13, 18). These subtypes include the luminal A and B, HER2-overexpressing (Human Epidermal Growth Factor Receptor 2), normal-like, and basal-like breast cancers (13). Each subtype is associated with a distinct prognosis and subsequent clinical outcome that makes identification of each patient tumor subtype both necessary and relevant (19).

Luminal breast cancers makeup approximately 45% of all breast cancers and are most likely to be low grade and differentiated (20). Identification of luminal tumors is carried out by staining for keratins 8/18, which are known to be expressed in normal luminal cells (18). Additionally, these tumor types express the estrogen (ER) and progesterone (PR) receptors and while luminal tumors are generally positive for these hormone receptors, variation in their expression leads to the classification of the luminal A and B groups (8, 13). Several genes, known to encode transcription factors that interact with ER, are upregulated in luminal-type breast cancers (20). These genes include FOXA1, GATA3, and PAX2, among others (18, 20). Some of these transcription factors serve as the driving forces behind luminal breast cancer proliferation and survival (20, 21). Despite intergroup heterogeneity, luminal tumors are clinically more easily treatable than HER2 or basal-like tumors and are generally associated with a good prognosis (22).

HER2/neu is overexpressed in 25% of all breast cancers as a result of amplification of the ERBB2 gene (18, 23). HER2-overexpressing tumors express ER to a lesser degree than luminal tumors (18). In fact, these tumors are more similar to basal-like tumors in that they lack expression or upregulation of those genes known to characterize ER positive tumors (18). HER2 overexpression is associated with tumors that are less differentiated and have a higher extent of proliferation, invasion, and metastasis (24). Clinically, HER2 overexpression correlates with a poorer prognosis and increased mortality rate in comparison to luminal tumors (25).

Finally, the basal-like breast cancers represent about 15% of all breast cancer cases and are identified by staining for keratins 5/6 or 17 (18, 19, 26). While most of these tumors share histological features, they are difficult to define universally due to the lack of common morphological features used during diagnosis (19, 20). As the name suggests, basal-like carcinomas express gene patterns analogous to those seen in the normal basal, or myoepithelial cells, of the breast (13, 26). Notably, basal breast cancers are associated with the “triple-negative” phenotype, meaning they lack ER and PR, and may exhibit little or no expression of the HER2 growth factor receptor (8). Interestingly, basal carcinomas have also been associated with the $CD44^{+}/CD24^{-/low}$ expression pattern and this is virtually unseen in the other subtypes (27). $CD44^{+}/CD24^{-/low}$ cell populations have been linked to being less differentiated and having higher invasive capabilities, a property that reflects the poor patient outcome seen in the clinic (19, 27). Basal-like breast cancers generally develop in patients at a younger age, are aggressive, are likely to metastasize, have a poor prognosis, and are characterized by a high mortality rate (26).

Breast Cancer Staging

Patients who have breast cancer are diagnosed at various points during disease progression. Cancer staging has evolved as a way to convey the extent and severity of a patient's disease by describing the basic primary tumor characteristics and degree of spread from the original site, if any (9). Breast cancer staging is useful for clinicians because it provides them with a common terminology when referring to patient disease status (9). Furthermore, it provides a useful way to approximate prognosis, employ treatment strategies, and monitor disease progression (28).

During the mid 20th century, a detailed system of staging, termed the TNM staging system, was developed (28). This system describes those characteristics of cancer that are thought to most influence prognosis including tumor size, node involvement, and identification of metastases (tumor-node-metastasis) (28). The American Joint Committee on Cancer, AJCC, has since produced a TNM staging of breast cancer that has undergone constant revisions to reflect the updates in our knowledge of disease progression (28). The most current TNM staging utilizes clinical, pathological, recurrence, and autopsy classifications to obtain the most accurate and descriptive stage of a patient's disease (9, 28) (Table 1).

Table 1

Basic Breast Cancer Stages

Stage	Sub-Stage	Description
I	A	Tumor \leq 2cm, no spread
	B	<ul style="list-style-type: none"> - No tumor in breast, but cancer cells in lymph nodes - Tumor \leq 2cm and cancer cell clusters in lymph nodes
II	A	<ul style="list-style-type: none"> - No tumor in breast, cancer in axillary lymph nodes - Tumor \leq 2cm, axillary lymph node spread - 5cm \geq Tumor \geq 2cm, no axillary lymph node spread
	B	<ul style="list-style-type: none"> - 5cm \geq Tumor \geq 2cm, axillary lymph node spread - Tumor \geq 5cm, no axillary lymph node spread
III	A	<ul style="list-style-type: none"> - No tumor in breast, cancer in axillary lymph nodes or in lymph nodes near breastbone - Tumor \leq 2cm, cancer in axillary lymph nodes or in lymph nodes near breastbone - 5cm \geq Tumor \geq 2cm, cancer in axillary lymph nodes or in lymph nodes near breastbone - Tumor \geq 5cm, cancer in axillary lymph nodes or in lymph nodes near breastbone
	B	<ul style="list-style-type: none"> - Tumor any size, spread to chest wall and/or skin - Possible spread to axillary lymph nodes or lymph nodes near the breastbone
	C	<p>No sign of cancer or tumor of any size that may have spread to chest wall and/or skin:</p> <ul style="list-style-type: none"> - Possible spread to lymph nodes near collar bone - Possible spread to axillary lymph nodes or lymph nodes near breastbone
		Operable: Cancer found in 10+ axillary lymph nodes, in lymph nodes below collarbone, in axillary lymph nodes and in lymph nodes near the breastbone
		Inoperable: Cancer has spread to the lymph nodes above the collarbone
IV		Cancer has spread to other organs, most commonly the bones, lungs, liver, or brain.

Reference (9)

Treatment Strategies

The therapeutic significance of the heterogeneous nature of breast cancer is that each patient case must be assessed individually and a therapy regimen should be designed based on the results of that assessment (29). Therapeutic decisions rely heavily on proper staging and analysis of a patient's disease (30).

Surgery is often the standard mode of treatment for patients with *in situ* carcinomas or very early stage invasive carcinomas (30). Radical mastectomies have been replaced by two types of breast-conserving surgeries (30). First, patients with small, non-invasive or early stage invasive breast cancers may undergo lumpectomy followed by radiation therapy (30, 31). Second, in the case of patients who have larger primary tumors and local advancement of their disease, chemotherapy administration prior to surgery, also known as neoadjuvant therapy, has proved to benefit some patients by causing tumor shrinkage and facilitating its removal (30). Furthermore, the newly developed method of sentinel lymph node mapping, whereby a blue dye is administered to the surrounding area of a tumor, has reduced the need for axillary lymph node dissections because the histological state of the sentinel lymph nodes is highly predictive of the state of the axillary lymph nodes (32). Disease-free sentinel lymph nodes spare the removal of the axillary lymph nodes which is associated with increased patient morbidity (32).

In contrast to taking a surgical approach, primary tumors may also be treated systemically with hormonal agents, various combinations of chemotherapies, or both (31). The treatment that is administered depends on the hormone receptor status of the tumor, identifying markers, and the extent of the disease (30-32). Examples of agents

used in the treatment of ER and PR positive breast cancers include tamoxifen, toremifene, fulvestrant, and progestins (30). For patients with a receptor positive status, these hormone therapies may be used in combination with other chemotherapies (30). Targeted therapy also exists for patients with HER2 overexpression including, but not limited to, trastuzumab and lapatinib (25). Trastuzumab may also be used for patients with hormone positive disease (30). Those patients whose breast cancer is characterized as triple-negative are harder to treat because the pathways that drive tumor growth under these conditions haven't been fully elucidated (33). Treatment is varied and often includes the use of platinum-based drugs, taxanes, cetuximab, imatinib, pertuzumab, and others (33). Chemotherapy is generally the primary therapy administered, but it can also be used in combination with radiotherapy, and again, the decision to utilize this mode of treatment is dependent on the characteristics of the patient's disease (31).

If a patient's disease is determined to be metastatic at the time of diagnosis, surgery is usually not the primary choice of therapy because the cancer has already spread and cannot be contained by removal of the tumor, although it can be beneficial in rare cases (32). At this point, it is important to identify the number and locations of the metastases in order to develop an appropriate therapeutic protocol (32). Breast cancer preferentially metastasizes to the lungs, bone, brain, and liver (32). Metastatic patients receive several chemotherapeutic and occasionally hormonal agents in addition to radiotherapy, but often their disease becomes resistant to the available therapies and the patient ultimately succumbs to their cancer (32, 33). Most metastatic breast cancer cases are triple-negative (33). As aforementioned, there currently isn't a recommended systemic treatment regimen specific for triple-negative cancers (33). Therefore, there is

motivation to find molecular therapeutic targets that are necessary and sufficient for progression of the cancer to the malignant state in order to broaden the treatment options available to these patients (33).

Introduction to the Eph Subfamily and EphA2

EphA2 is a member of the largest subfamily of receptor tyrosine kinases (RTKs), the Eph subfamily (34). EphA1, the first receptor in the Eph subfamily to be discovered, was identified in 1987 by Hirai *et al* (35). To date, there are fourteen total members that are further subdivided into the EphA and EphB subgroups based on sequence homology (36). The EphA subgroup has nine members, EphA1-8, and the EphB subgroup has five members, EphB1-6 (36).

Eph receptors bind membrane-bound ligands on adjacent cells, called ephrins, that are also subdivided into the A and B groups based on the receptor they bind (37). There are five A-ephrin ligands that are anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage (37). In contrast, the B-ephrins are transmembrane ligands consisting of only three total members (37). In general, EphA receptors preferentially bind A-ephrins, while EphB receptors preferentially bind B-ephrins, although there is some binding promiscuity seen between receptors and ligands of opposing groups (38). Despite this, all ephrin-Eph receptor binding leads to receptor autophosphorylation and subsequent activation (34, 39).

Ephrin-Eph receptor interactions have been linked to several functions, mostly in the developing nervous system where they are involved in axon guidance, cell migration, synaptic plasticity, and boundary maintenance (38, 39). Furthermore, there are also

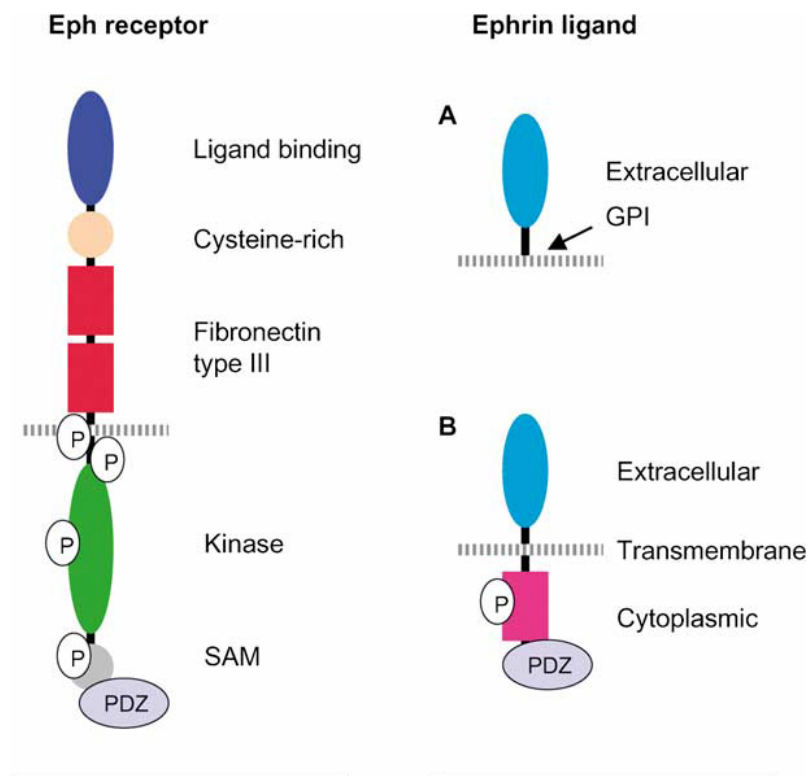
implications of Eph-ephrin involvement outside of the nervous system such as during vascular development (39, 40). While their functions are numerous and diverse, studying the effect of Eph-ephrin signaling has been difficult due to the functional compensatory ability of the receptor-ligand complexes (39).

Located on chromosome 1p36.1, EphA2 is a 130 kDa receptor tyrosine kinase that was discovered in 1990 and was originally named *eck*, epithelial cell kinase, because of the abundance of its mRNA and protein expression in epithelial cells (41, 42). The entire EphA2 gene consists of an open reading frame flanked by either a 5' or 3' untranslated region (41). The open reading frame encodes for 976 amino acids and the 5' and 3' regions are composed of 113 and 925 nucleotides, respectively (41). Original *eck* expression analysis revealed that those tissues with the highest levels of EphA2 include the skin, ovary, small intestine, and lung (41). Subsequent studies have revealed the expression and importance of EphA2 function in the breast (43).

Extracellularly, the EphA2 receptor is composed of a NH₂-terminal ligand-binding domain, cysteine-rich domain, and two fibronectin-type III repeats (44). The intracellular domain begins with a juxtamembrane domain (44). This domain has two tyrosines that can carry out autophosphorylation (44). Next there is a tyrosine kinase domain, sterile α motife (SAM), and PDZ domain-binding motif (44). The latter two, located at the COOH-terminal end, interact with proteins that can initiate downstream signaling such as the src-like adapter protein (SLAP), c-cbl, p85 subunit of phosphoinositide 3-kinase (PI3K), src-homology domain-containing (SHC) protein, SH2 domain-containing tyrosine phosphatase 2 (SHP2), and focal adhesion kinase (FAK) (34, 44, 45) (Figure 3).

Figure 3

Structure of Eph Receptors and Ephrin Ligands



Reference (46)

Permission to Use Figure Granted by Original Publisher, Elsevier

EphA2, like its family members, also participates in embryonic development of the central nervous and vascular system (47). Specifically, it has been identified to be important in pattern formation, a process that occurs during gastrulation to ensure correct cell differentiation and growth within the embryo, as well as with proper development of various tissues within the fetus (48). Additionally, EphA2 is important in correct placental development during the first trimester of pregnancy (49).

In addition to its expression and involvement in prenatal functions, EphA2 also plays a crucial role in the young adult and adult organism, where its expression is limited to proliferating epithelial cells (43, 50). Mammary branching morphogenesis is a complex process that occurs in the breast shortly after the onset of puberty whereby the branched ductal network is established (43, 51). EphA2 was found to be the only Eph family receptor expressed in the terminal end buds, structures known to initiate the branching process in the breast (43, 51). Post puberty, the expression of EphA2 is upregulated during the pre-estrous and estrous phases of the estrous cycle (50, 52). Importantly, EphA2 expression is significantly decreased in differentiated mammary epithelium (53). This unique expression of EphA2 in the breast and its overexpression in highly aggressive breast cancers is one of many reasons why this receptor tyrosine kinase has been a protein of research interest and investigation (53, 54).

EphrinA1 Ligand –EphA2 Receptor Interactions

Ephrin ligands and Eph receptors have a unique relationship in regards to their interaction and function in normal versus cancerous cells. As previously mentioned, ephrinA ligands preferentially bind EphA receptors, and the same can be said for B

ligands and receptors (44). This is due to the specific amino acids found in the G-H loop of ephrins, the location where receptor and ligand binding is thought to occur (44). Slight differences in the amino acid sequence unique to certain members of the A or B receptors, such as the EphA4 receptor, are responsible for the promiscuity of binding between the A and B groups (44).

It is important to remember that both the ephrin ligands and the Eph receptors are membrane bound (34). Therefore, cell-cell contact is necessary for any ligand to interact with its receptor (34). An ephrin ligand will bind its receptor through a high-affinity interface which subsequently causes a lower-affinity interface to be exposed (34, 55). This lower-affinity interface allows for tetramerization of two ephrin-Eph complexes (34, 55). Transphosphorylation of the cytoplasmic portion of an Eph receptor occurs on the tyrosine residues in the juxtamembrane region, releasing the inhibition of the kinase domain and allowing for receptor activation (34, 55).

One of the more interesting features of the EphA2 receptor is its participation in both tumor suppressive and tumor promoting functions (50, 56). In breast cancer, EphA2's tumor promoting abilities are associated with being unbound to ligand, whereas its tumor suppressive functions result from ligand activation of the receptor (56). This further translates into differences in the tyrosine phosphorylation state of the EphA2 receptor (56). Ligand stimulation causes receptor phosphorylation that initiates a variety of downstream activities that negatively regulate migration, invasion, and growth in non-transformed epithelial cells (56). For example, when activated by ligand, EphA2 will interact with molecules like the SHC protein, GRB2 (Growth Factor Receptor Bound Protein 2), SLAP, and will initiate PI3K activity (45, 57, 58). This is to be contrasted

with the cancerous state, where EphA2 is not ligand activated and therefore exhibits significantly reduced tyrosine phosphorylation at sites that would normally be phosphorylated in response to ligand stimulation (56). Despite this lack of receptor stimulation by ligand, EphA2 maintains its enzymatic activity (56, 59). The downstream pathways initiated by EphA2 in its non-ligand bound state are distinct from its ligand-activated state and result in the promotion of migration, invasion, and growth (53, 56, 59). For example, EphA2 has been reported to interact with FAK and E-Cadherin independent of ligand activation (60). Loss of E-cadherin disrupts ligand-receptor binding and increases EphA2 association with FAK, ultimately leading to increased ECM interactions (59, 60).

In summary, the disparity in tyrosine phosphorylation in non-neoplastic and metastatic cells has important downstream signaling consequences that result in either the maintenance of a homeostatic state or promotion of cancer progression. These consequences have not been fully explored, but further study and enhanced understanding of them would allow for better therapies to be developed for EphA2 overexpressing breast cancers.

The Role of EphA2 in Cancer

In addition to the many functions of EphA2 in the normal processes of embryogenesis and in adult epithelial tissues, overexpression of EphA2 has been found in a variety of aggressive tumor types and is sufficient to drive malignant transformation in non-transformed cell lines *in vitro* (53, 61, 62).

Zeng *et al.* sought to look at EphA2 levels in both cancerous and benign prostate samples (63). Immunohistochemical analysis revealed high intensity EphA2 staining in both high-grade intraepithelial neoplasia and adenocarcinomas of the prostate (63). Conversely, adjacent benign tissue from the same samples showed significantly weaker staining patterns for EphA2, easily differentiating the neoplastic regions from the normal regions (63). This study was particularly significant because the expression of EphA2 in high-grade neoplastic prostatic epithelial cells, a pre-malignant condition, implies that EphA2 is involved in the early stages of disease progression (63).

EphA2 involvement has also been implicated in pancreatic cancer, the fourth deadliest cancer in the United States (64). Overexpression of EphA2 in the Capan2 pancreatic cell line, a cell line that is poorly metastatic *in vivo* with low natural levels of EphA2, conferred an increase in invasive capability *in vitro* and increased resistance to anoikis (64). Furthermore, EphA2 siRNA was used to suppress EphA2 overexpression in the more aggressive PANC1 and MIAPaCa2 cell lines (64). This resulted in a decrease in FAK phosphorylation, a molecule whose activation is important in EphA2-mediated interactions with the extracellular matrix (ECM) during cancer progression (64).

Additionally, EphA2 is commonly overexpressed in ovarian cancer (65). This overexpression is related to more severe forms of the disease and is associated with poor patient outcome (65). It has been revealed that a possible reason for this negative outcome in EphA2 overexpressing ovarian cancer patients is due to the involvement of the receptor in the process of angiogenesis (66). Angiogenesis is one of the hallmarks of cancer that is largely responsible for tumor sustenance and growth (66, 67). Targeting EphA2 in ovarian cancer has been the subject of much investigation (66).

Prostate, ovarian, and pancreatic cancer are only a few examples where EphA2 overexpression is known to play a role in tumor promotion and aggressive behavior (63, 65, 66). Others include esophageal cancer, melanomas, colorectal cancer, cervical cancer, renal cancer, and non-small cell lung cancer (68-73).

Regulation of EphA2

Due to the association of EphA2 with wide-spread involvement in the most aggressive cancers, there has been interest in elucidating the mechanisms that regulate it in a normal environment. Cancer-causing mutations result in the disruption of molecular regulatory pathways that are necessary for normal cell function and homeostasis (67). The unveiling of regulatory mechanisms that influence EphA2 expression has provided insight into how this protein becomes overexpressed in breast cancer and has opened the door for investigation of the effects of this aberrant upregulation.

Estrogen & c-myc

As previously mentioned, a large majority of the most aggressive breast cancers do not express the estrogen receptor (8, 74). Furthermore, EphA2 is largely overexpressed in aggressive and ER negative cell lines (53). Zelinski *et al* confirmed that there is an inverse relationship governing ER and EphA2 expression at the protein level (75). This observation led to the hypothesis that estrogen is responsible for the negative regulation of EphA2 expression (75). Indeed, multiple non-transformed mammary epithelial cell lines were used to show a dose-dependent decrease in the protein levels of

EphA2 (75). Furthermore, estradiol treatment of cell lines deficient in ER expression had no effect on EphA2 expression (75). An interesting finding in this investigation was that two transformed cell lines had limited expression of both ER and EphA2 (75).

Ultimately, it was discovered that independently high levels of c-myc, a gene target of estrogen, could also cause inhibition of EphA2 expression (75, 76). Taken together, these results suggest that as breast cancer progresses towards malignancy, ER expression is lost and this releases EphA2 from the inhibitory effects of estrogen, allowing it to become overexpressed on the cell membrane (75, 76).

Ras

Furthermore, Macrae *et al* have shown that stimulation of the Ras/MAPK pathway by growth factor receptors initiates transcription and upregulation of EphA2 at the RNA and protein level (77). Utilization of two MEK inhibitors, U0126 and PD98059, resulted in a decrease in EphA2 protein levels in a variety of breast cancer cell lines, further confirming that this receptor tyrosine kinase is downstream of the MAPK pathway (77).

An important result of this study was the observation that expression of EphA2 receptor and ephrinA1 ligand is mutually exclusive in breast cancer cell lines (77). EphA2 is overexpressed in the aggressive, mesenchymal-like lines whereas ephrinA1 is expressed in the less aggressive, more epithelial-like lines (77). One explanation for the lack of EphA2 in ephrinA1 expressing cells is that ligand activation of EphA2 causes receptor internalization and subsequent degradation (77).

Interestingly, just as the MAPK pathway stimulates EphA2 expression, it is simultaneously inhibiting ephrinA1 expression, offering an explanation as to why EphA2 overexpressing cells lack ephrinA1 (77). Though these cells are not expressing ligand, EphA2 can be activated by ligand presented on ligand expressing cells, leading to receptor down regulation (77) (Figure 4).

One exception to this relationship is the MDA-MB-231 breast cancer cell line that has an activating mutation in K-Ras and a B-Raf mutation (77-79). These mutations make MDA-MB-231 unresponsive to the effects of ligand activation and subsequent MAPK pathway inhibition (77).

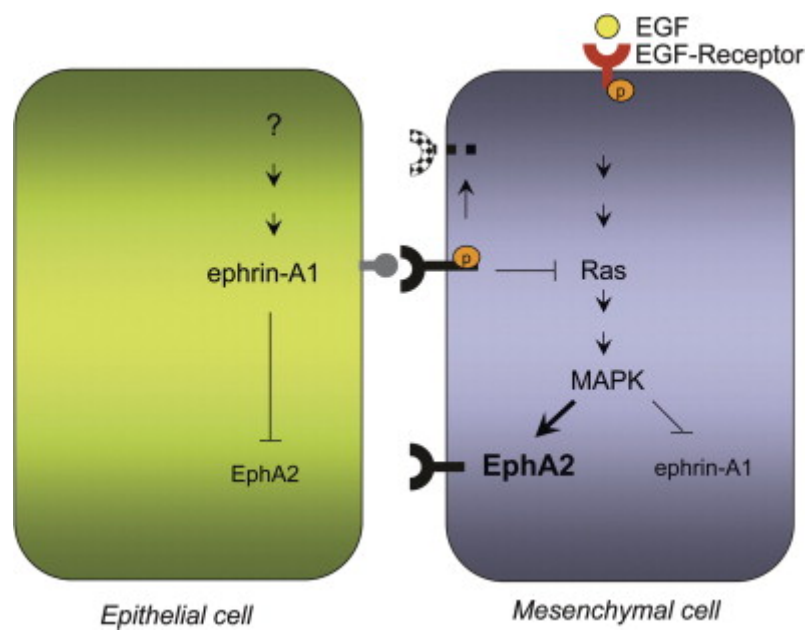
E-Cadherin

Since EphA2 and ephrinA1 are both bound to the plasma membrane and require cell-cell contact to interact, it was postulated that E-cadherin, a molecule important in mediating adhesion between epithelial cells, might have a role in regulating EphA2 receptor ligand-dependent activation (59, 80).

A comparison of EphA2 cellular localization in nontransformed mammary epithelial cells and metastatic cells revealed markedly different distributions of the receptor (59). In nonneoplastic cells, EphA2 was localized to those areas where cells were coming into close contact (59). However, EphA2 in metastatic cells was localized to the membrane ruffles of migratory cells or it displayed a dispersed pattern without localization to any specific area (59). Subsequent studies revealed that not only do EphA2 and E-cadherin colocalize to the points of cell-cell contact, but disruption of E-cadherin adhesions resulted in a decrease in the amount of phosphorylated EphA2 and the receptor

Figure 4

Ras Regulation of EphA2 and EphrinA1 Expression



Reference (77)

Permission to Use Figure Granted by Original Publisher, Elsevier

then became diffusely distributed, as seen in transformed cells (59). Furthermore, EphA2 overexpression in metastatic cells respond to induced expression of E-cadherin by becoming phosphorylated and localizing to cell-cell adhesion locations (59).

Overall, E-cadherin is thought to influence ligand-dependent activation of EphA2 by stabilizing epithelial cell-cell interactions (59). This stabilization permits binding between EphA2 and ephrinA1 and functions to maintain normal levels of the receptor in epithelial tissues (59). It's important to note that a high cell density is not sufficient to cause EphA2 phosphorylation and subsequent receptor downregulation, rather, E-cadherin is the necessary component that creates the strong adhesions between cells and allows for ligand-receptor interaction (59).

P53

p53 is mutated in a large majority of all cancers (81). Considering that p53 normally functions to control cell cycle arrest and cell death, it's not surprising that deviation from this regulatory ability would cause havoc on normal cell proliferation and could ultimately lead or contribute to cancer development (81, 82).

EphA2 contains a binding site for wild type p53 in its promoter indicating that p53 is a regulator of EphA2 expression at the transcript and protein levels (83). In response to DNA damage, EphA2 tyrosine phosphorylation and upregulation results from p53 activation, leading to EphA2-mediated inhibition of cell growth and promotion of apoptosis (83). It is possible that p53 activating mutations could function to increase protein levels of EphA2, contributing to the overexpression of the receptor in aggressive breast cancers (56, 83).

Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP)

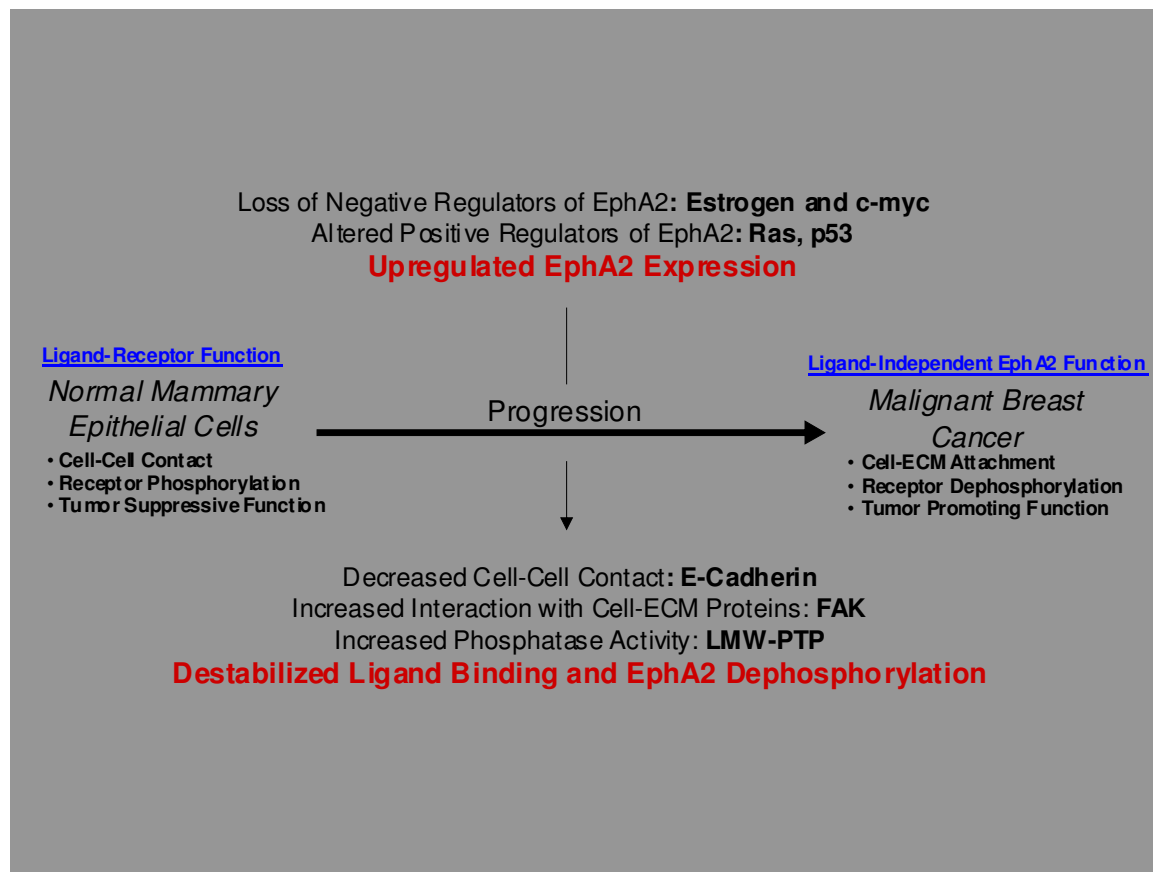
It is curious that despite the overexpression of EphA2 in some of the most aggressive breast cancers, there is limited receptor tyrosine phosphorylation (53, 56, 59). Investigation into the mechanism behind the lack of receptor phosphorylation in transformed cells has led to the identification of one particular phosphatase, Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) (84).

Disruption of ligand binding by treating cells with the chemical agent ethylene glycol tetraacetic acid (EGTA) or by dominant-negative inhibitors resulted in suppressed phosphotyrosine content (59, 84). Furthermore, addition of phosphatase inhibitors after treatment of non-neoplastic cells with EGTA hindered the reduction in phosphorylated EphA2 (84). Subsequent treatment of breast cancer cells with phosphatase inhibitors alone resulted in an increase in the EphA2 phosphotyrosine content (84).

These studies led to the identification of LMW-PTP, the phosphatase that regulates EphA2 tyrosine phosphorylation. LMW-PTP is highly overexpressed in a variety of malignant cell lines, with breast cancer lines being no exception (84). It functions to dephosphorylate EphA2, thereby stabilizing the protein at the cell surface (84). The oncogenic activity of LMW-PTP depends on this stabilization of non-phosphorylated EphA2 (84).

Figure 5

Summary of EphA2 Regulation



Reference (50)

Involvement of EphA2 in Metastatic Breast Cancer

Metastasis

The sequential steps that must be carried out by primary tumor cells in order for them to successfully disengage from the initial tumor site and develop disease at distant locations are both numerous and complex (85). “Metastasis” is the name given to describe this process of disease dissemination to secondary, or more, locations from the primary tumor (85). Metastasis involves transformation of cells at the primary site, neovascularization, intravasation of transformed cells into circulation, adherence to vessels in the secondary location, extravasation of the transformed cells into the surrounding organ, response to stimulatory environmental factors, and finally, growth within the secondary site (85-87). All of these steps must be completed for metastatic success (88). Interestingly, studies have shown that entrance into the circulation by cells from mammary adenocarcinomas was relatively common, but less than 0.01% of circulating cells survive to develop tumors at metastatic sites (89, 90). This opens debate about the existence of a select population of cells from the primary tumor that are predisposed to successfully metastasize (89, 91). However, despite the seemingly inefficient nature of the metastatic process, metastasis remains the primary contributor to high cancer patient mortality rates, including those with breast cancer (85, 92, 93).

EphA2 Involvement in Breast Cancer

The RTK EphA2 is a molecule of interest in the study of many cancers, but has not been fully investigated in the breast cancer setting. Despite this, there is sufficient evidence to support that such a study would be worthwhile and beneficial.

First, EphA2 has been shown to be overexpressed in both breast cancer cells *in vitro* and in clinical specimens (53). More significant is that this aberrant overexpression of EphA2 is seen only in the triple-negative, basal breast cancer subtype, which are aggressive and highly metastatic (7, 26, 33). Furthermore, EphA2 overexpression is sufficient to cause transformation of non-transformed mammary epithelial cells (53). The significance of these findings is that EphA2 is not simply acting as a marker of tumor progression, rather, it is a direct player in malignant transformation (56).

Second, there is a deficit in the amount of available therapies for patients with triple-negative breast cancer (33). Since EphA2 has been shown to have a dominant role in the progressed state of this disease, investigation into the extent of its participation in metastasis is certainly an area of interest (33, 53). Furthermore, EphA2 expression in the developed mammary gland is significantly downregulated in comparison to the extent of overexpression it exhibits in breast cancers (53, 54). Therefore, it seems likely that therapy designed to target EphA2 would cause minimal harmful effects to the surrounding, normal mammary tissue.

Finally, EphA2 is regulated by molecules already associated with advanced stage cancers. Examples of such molecules include E-cadherin and ER (59, 75). Loss of cell-cell contact due to the disruption of E-cadherin mediated adhesions has been implicated as a mechanism that facilitates the migration and invasion of tumor cells (59). In addition,

hormone receptor status is one of the most important features of a patient's breast cancer because of the prognostic ability (75). Upregulation of EphA2 is associated with a deregulation in both ER expression and E-cadherin expression yet, the extent of tumor dependence on EphA2 overexpression in metastatic breast cancers hasn't been completely elucidated. Exploring the effects of reducing EphA2 in an attempt to investigate the biology associated with this receptor's involvement in the metastatic process would contribute to the greater understanding of the progressive mechanics of breast cancer.

Specific Aims of the Project

The first specific aim of this project was to stably reduce EphA2 receptor levels in breast cancer cell lines. The purpose of this aim was to provide proof of the principle that when EphA2 expression is low in breast cancer cells, they exhibit a decrease in metastatic potential *in vitro* and negatively affect tumor growth and development of metastases *in vivo*.

The second specific aim of this project was to transiently reduce EphA2 levels in breast cancer cells using siRNA. The purpose of this aim was to demonstrate that EphA2 can be reduced in breast cancer cells and in established mammary fat pad tumors and this reduction has a negative affect on properties of metastasis *in vitro* and tumor growth *in vivo*.

Table 2

**Summary of Notable Characteristics of the Primary Cell Lines Used in
this Thesis Research**

Cell Line	Characteristics
MDA-MB-231	<ul style="list-style-type: none">- KRAS Mutation- BRAF Mutation- ER Negative- Vimentin Positive- EphA2 Expression
BT549	<ul style="list-style-type: none">- ER Negative- Vimentin Positive- EphA2 Expression
HCC 1954	<ul style="list-style-type: none">- HER2/Neu Overexpression- ER Negative- PR Negative- EphA2 Expression

Reference (94)

- Chapter 2 - Materials & Methods

Materials & Methods

Cell Lines

MDA-MB-231 cells were obtained from Dr. Relda Cailleau's laboratory at the M.D. Anderson Cancer Center. These cells were originally isolated from the pleural effusions of a breast cancer patient of Caucasian descent. A variant of the original cell line was established from metastatic lesions in a nude mouse that had been injected with MDA-MB-231. These variant cells were then transduced with a lentivirus vector expressing luciferase (Lentigen, Baltimore MD). BT549 and HCC1954 were received from the American Type Culture Collection. HCC1954 cells were originally isolated from an East Indian, female patient with TNM stage IIA ductal carcinoma. BT549 cells were originally isolated from a Caucasian, female patient with ductal carcinoma. See Table 2.

Cell Culture Conditions

MDA-MB-231 Luc cells were maintained in monolayer culture at a temperature of 37°C and 5% carbon dioxide (CO₂). Minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 1% 100mM sodium pyruvate (GIBCO), 1% non-essential amino acids (GIBCO), the antibiotics penicillin and streptomycin (GIBCO), L-glutamine, and vitamins was used to culture these cells. BT549 cells were maintained in monolayer culture at a temperature of 37°C and 5% CO₂. Dulbeccos's modified eagle medium (DMEM) supplemented with Ham's F-12 nutrient mixture, 10% FBS, L-glutamine, and penicillin/streptomycin was used to culture these cells. HCC1954 cells were maintained in monolayer culture at a temperature of 37°C and 5% CO₂. DMEM-High Glucose

(DMEM-HG) supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin was used to culture these cells.

Preparation of Whole Cell Lysates

Cells were plated in a 6 well plate to achieve a next-day confluency of 70-80%. After aspirating the media, cells were washed once with PBS and this was then aspirated as well. 10 µl of 10 mg/ml stock PMSF in isopropanol and 10 µl of 100 mM stock sodium orthovanadate (Na_3VO_4) in addition to a Mini, EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics) were freshly added to 1 ml of RIPA buffer (50 mM Tris, 150 mM sodium chloride, 0.1% SDS, 0.5% Sodium Deoxycholate, Triton X 100) and then 100 µl this mixture was added to each well. The 6 well plate was put on ice so that each well could be scraped with a scraper (Corning Incorporated, Costar® 3010), where the well contents were then transferred into an appropriately labeled microcentrifuge tube. The collected protein lysates were allowed to incubate on ice for 30 minutes to 1 hour. After incubating for the specified time, the microcentrifuge tubes containing the protein lysates were transferred to a centrifuge where they were spun for 20 minutes at 10,000 rpm at a temperature of 4°C. The supernatant was collected and transferred to a separate, sterile microcentrifuge tube where the protein concentration was then determined using a Biorad Protein Assay kit and its protocol in addition to the use of a DU®-65 Spectrophotometer.

Immunoblot Analysis

20 µg of protein collected from the whole cell lysates was combined with Laemmli Buffer (Sigma) and boiled for 5 minutes. Protein samples were then separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) set at 100 volts. After separation, protein was transferred to a nitrocellulose membrane (Biorad) also set at 100 volts. Membranes were blocked using 5% milk in TBST. The following describes the primary antibodies used to probe the nitrocellulose membrane and the specific conditions required for each: Anti-Eck/EphA2 (Millipore, Cat#05-480) diluted 1:1000 in 5%FBS-TBST, anti-EGFR (Upstate, Cat#06-847) diluted 1:2000 in TBST only, anti-actin (Sigma, Cat#A2066) diluted 1:5000 in 1%FBS-TBST. The following describes the secondary antibodies used: Anti-rabbit IgG (Cat#NA934V) and anti-mouse IgG (Cat#NA931V) secondary antibodies (Amersham Life Science ECL Western Blotting Analysis System). GE Healthcare ECL Western Blotting Detection Reagents (Cat#RPN2106) were used for detection.

Isolation of RNA and Quantitative Real Time Polymerase Chain Reaction

Cells were plated in a 6 well plate and grown to 70-80% confluency. RNA was isolated using the Array Grade Total RNA Isolation Kit (SA Biosciences, Cat#GA-013) according to its protocol. Isolated RNA was then treated with TURBO DNA-free reagents (Ambion, Cat#AM1907) and made into cDNA by the process of reverse transcription using the High Capacity DNA Archive reagents (Applied Biosystems). Each test well on the PCR plate had a total reaction volume of 25 µl which was a mixture of 1.25 µl of 20X Gene Expression Assay Mix (Applied Biosystems), 12.5 µl of 2X TaqMan Universal PCR

Master Mix (Applied Biosystems), and 11.25 µl of 200 ng cDNA that was diluted in RNase-free water. Duplicates of each sample were tested with 18S in each sample tested as the intrinsic positive control. After plate preparation, the 7500 Real-Time PCR System (Applied Biosystems) was used to carry out the reaction and data was converted into cycle threshold (Δ CT) measurements. qRT-PCR was performed for EphA2 and EGFR in this way.

siRNA Transfection

Cells were grown in a 6 well plate to 70-80% confluency. The next day, transfection mixtures were prepared. Control cells were treated with 400 µl of serum-free medium only. Control cells for the transfection reagent were treated with 400 µl of serum-free medium with 12 µl of HiPerFect Transfection Reagent (Qiagen, Cat#301705). Cells treated with the non-targeting siRNA were treated with 400 µl of serum-free medium, 12 µl of HiPerFect Transfection Reagent, and 1.5 µl of the negative control siRNA (Ambion, Cat#AM4613). Cells treated with EphA2-targeting siRNA #1 were treated with 400 µl of serum-free medium, 12 µl of HiPerFect Transfection Reagent, and 1.5 µl of the EphA2-targeting siRNA ID: 146479 (Ambion, Cat#AM16708A). Cells treated with EphA2-targeting siRNA #2 were treated with 400 µl of serum-free medium, 12 µl of HiPerFect Transfection Reagent, and 1.5 µl of the EphA2-targeting siRNA ID: 242385 (Ambion, Cat#AM16708). After preparation of transfection mixtures, culture medium was aspirated, mixtures were added to the appropriate wells, and cells were incubated for 5-6 hours. After incubation, 1.9 ml of the appropriate serum-containing medium was added to

each well and cells were incubated for 72 hrs at which point they would be used for various assays.

Migration Assay

Approximately 1×10^5 cells were plated into the upper, non-coated chamber of one of 24 well inserts with an 8 micron pore size (BD Biosciences, Cat#354578). Cells were plated in serum-free medium in the upper chamber and were placed in the lower chamber which contained either serum-free medium + 0.1%BSA or serum-free medium containing 0.1%BSA and EGF (Invitrogen, Cat#13247-05) as a chemoattractant. Migration plates were placed in the incubator and cells were allowed to incubate for 6 hours. Cells that did not migrate were wiped away with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed and stained with the Fisher HealthCare PROTOCOL Hema 3 Manual Staining System (Fisher Scientific Company L.L.C.). Membranes were allowed to dry before being cut away from the chamber and placed on microscope slides (Fisher Scientific, Cat#15-188-51) with PerMount (Fisher Scientific) and covered with a cover slide (Corning 24x40mm, Cat#2935-244). Migrated cells were then counted.

ELISA for VEGF

2.5×10^5 cells were plated in a 6-well plate. The next day, the medium was aspirated and 1ml of medium was added back in overnight and supernatant was collected the next day. VEGF capture antibody (R&D Systems, Cat#AF-293-NA) was added to a 96 well E1A plate (Costar 9018) the day before ELISA was performed. Wells were then washed with wash buffer (PBS+0.05%Tween 20) and blocking solution was added (PBS with 1%

BSA, 5% sucrose, 0.05% sodium azide) for 1 hour. Wells were washed with wash buffer. VEGF standards (R&D Systems, Cat#293-VE) and sample supernatants were added to appropriate wells for 2 hours. Wells were washed with wash buffer. VEGF detection antibody (R&D Systems, Cat#BAF293) was added to the wells for 2 hours. Wells were washed with wash buffer. Streptavidin HRP (R&D Systems, Cat#DY998) was added to wells for 20 min. Wells were washed with wash buffer. Substrate solution [1:1 mixture of color reagent A (H_2O_2) and color reagent B (tetramethylbenzidine), R&D Systems, Cat#DY999] was added to wells for 25 min. Stop solution (1M H_2SO_4) was added to the wells. Plate was read within 30 min. using the DU[®]-65 Spectrophotometer and VEGF concentrations were calculated based on standard curve values.

Co-Immunoprecipitation Assay

5×10^6 cells were plated in a 10 cm dish with 10 ml medium. When cells were 80% confluent, they were serum starved overnight and stimulated with 50ng/ml EGF for 15 minutes the next day. The medium was aspirated, cells were washed once with cold PBS, and 1ml of cold PBS was added to the dish to collect the cells. Cells were centrifuged at 4°C at 3000rpm for 5-10 minutes. The supernatant was dumped and cell pellets were lysed with 500 μl TNE buffer with inhibitors. Cell and buffer mixture was allowed to rotate for 1 hr at 4°C and then spun down at 12,000 rpm for 15 minutes at 4°C. The supernatant was transferred into a clean tube and the protein concentration was measured. Samples consisting of 20 μg of protein were prepared and set aside to be used for whole protein lysates control. 100 μg protein was taken from each protein sample and mixed with TNE buffer to take the total volume up to 1 ml. 5 μg of anti Eck/EphA2 antibody

(Millipore, Cat#05-480) was added to each sample and allowed to rotate at 4°C overnight. The next day, 25 µl of beads was taken per sample and washed with 1 ml TNE buffer three times. After washing, TNE buffer was added to the bead volume in a 1:1 ratio. 25 µl of the bead/TNE buffer mixture was added to each 100 µg protein sample and was allowed to rotate for 1-2 hrs at 4°C. Samples were spun down and beads were washed with 1 ml TNE buffer three times. 2x Laemmli buffer was added in a 1:1 ratio to the protein samples and also to the aliquots of 20 µg whole protein samples. All samples were boiled for 5 minutes. Proteins were then subjected to SDS-PAGE and western blot analysis to look at EGFR protein expression.

Lentiviral Delivery of shRNA for Stable Silencing of EphA2

Knockdown cell lines were made by using the Invitrogen website BLOCK-iT RNAi Designer to find shRNA sequences that had been ranked to indicate knockdown probability. Three highly rated EphA2 target shRNA sequences were selected: LV3: GGCTGAGCGTATCTTCATTGA; LV4: GCGTCATCTCCAAATACAAGC; LV5: GGTGATGAAAGCCATCAATGA. Sequence information was provided to the Viral Core Laboratory of the Department of Cancer Biology to prepare lentivirus with shRNA to EphA2. Sense and antisense EphA2-targeting oligonucleotides and a non-targeting control shRNA oligonucleotide (TTCTCCGAACGTGTCACGT) were created to have a hairpin structure and sticky ends. The EphA2 targeting and non-targeting shRNA oligonucleotides were then cloned into the pLVTHM expression vector (Addgene, Cat#12247). EphA2 shRNA knockdown lentivirus and non-targeting shRNA lentivirus were generated by transfecting the packaging plasmid (2nd generation including PAX2

and MD2G; Addgene, Cat#12259 and 12260) with the EphA2 expression plasmid into 293T cells. MDA-MB-231 Luc and BT559 cells were plated and grown to 70% confluency. Control cells were treated with 250 μ l of non-targeting control virus, 750 μ l of regular medium, and 3 μ g/ml of polybrene. Test cells were treated with 500 μ l of either LV3, LV4, or LV5 in addition to 500 μ l of regular medium and 3 μ g/ml of polybrene. Cells were incubated with the virus mixtures for 24 hours, after which the mixtures were aspirated and fresh medium was added. Cells were incubated and allowed time to grow. Cells were sorted at the M.D. Anderson Cell Sorting Facility based on expression of GFP.

Agarose Colony Formation Assay

Standard 2x MEM was prepared from 10x MEM liquid. 20 ml of the 10xMEM, 75 ml of double distilled H₂O, 2.9 ml of sodium bicarbonate (7.5% solution), and 2.1ml of pen-strep solution were mixed together. After mixing, the solution was filtered for sterilization and stored. To begin the assay, 1% and 0.6% agarose solution needed to be made from standard 2% agarose. The 2% agarose solution was warmed in the microwave and then mixed 1:1 with 2x MEM to make 1% agarose solution. In order to make 0.6% agarose solution, the 1% agarose solution was further diluted using serum-free MEM. 1ml of 0.6% agarose solution was added to each well of a 6 well plate, or according to how many samples there were including a duplicate. The 0.6% agarose solution was spread quickly and left in the hood to solidify. Cells were harvested and counted. The cell layer was mixed in proportions of 1% agarose, serum-free MEM, FBS (either to make 10% or 1% FBS), and cell suspensions in order to achieve a concentration of 0.3%

agarose, 10% or 1% FBS, and 1×10^3 cells in a final volume of 1.5 ml. 1.5 ml of each cell solution is now added to the appropriate well on top of the 0.6% base agarose layer. The plates were allowed to sit at room temperature for 30 min-1 hr before being put in the incubator for approximately 14-21 days. Every couple of days, a small amount of medium (0.5 ml) was added to the agarose cultures to prevent the gel from drying.

Immunocytochemistry for Fluorescent and Confocal Imaging

Cells were plated on chamber slides at 7×10^4 cells per chamber and incubated overnight. The next day cells were fixed with acetone, washed with PBS, and blocked with protein block (4% Fish gelatin in PBS, supplied by the Core Facility). EphA2 antibody (Millipore, Cat#05-480) was applied to cells and incubated in the cold room overnight. Cells were washed, protein blocked, and secondary antibody was applied for 1 hour. Alexa 488 goat-anti-mouse (MolPrb, Cat#A11029) secondary antibody was used to stain cells for fluorescent imaging and cy3 goat-anti-mouse secondary antibody was used to stain cells for confocal imaging (Jackson ImmunoResearch, Cat#111-166-047). Cells were washed, protein blocked, and the EGFR primary antibody (Santa Cruz, Cat#sc-03) was applied overnight in the cold room. Cells were washed, protein blocked, and EGFR secondary antibody was added for 1 hour at room temperature. Alexa 594 anti-rabbit secondary (Invitrogen, Cat#A11037) was used to stain cells for fluorescent imaging and cy5 donkey-anti-rabbit secondary (Jackson ImmunoResearch, Cat#711-176-152) was used to stain cells for confocal imaging. Cells were washed and counterstained with Hoescht (for fluorescent imaging of nuclei; Invitrogen, Cat#H3570) or cytox green (for

confocal imaging of nuclei). Cells were washed, slides were mounted with propylgallate, covered, and stored in the cold room protected from light.

Animals

Female athymic nude (NCR-*nu/nu*) mice were purchased from the Charles River Laboratory (Wilmington, MA) when they were four to five weeks old. The animals were housed in a facility that is approved by the American Association for Accreditation of Laboratory Animal Care.

Mammary Fat Pad Tumor Growth

Each experimental group consisted of 5-10 mice. Mice were anesthetized using Metofane (Pitman Moore, Inc.; Washington, NJ). Once anesthetized, an incision approximately 5 mm in length was made in the skin covering the lateral thorax to expose the mammary fat pad. A 27 gauge needle was used to inject 5×10^6 cells in 0.1 ml PBS into the mammary fat pad of each animal.

Experimental Metastasis

1×10^6 breast cancer cells suspended in a volume of 0.2 ml PBS were injected intravenously (i.v.) in the lateral tail vein of each mouse. A total of 5-10 mice were used per experimental group. Animals were watched closely until they were approaching death or until 10 weeks post injection. At either of these time points, the animals were sacrificed and the lungs were collected to be examined for metastases. Additionally, since the MDA-MB-231 Luc cells contain a luciferase gene, animals were monitored for lung

metastases approximately three weeks post-i.v. injection of the cells. Mice were injected i.p. with 0.2 ml of 15 mg/ml luciferin and imaged for bioluminescence using a Xenogen IVIS Imaging System. The extent of bioluminescence could be measured using the ROI (Region of Interest) tool provided in the imaging software.

Liposomal and Chitosan Nanoparticle Treatment

Cells that had been injected into the mammary fat pad were allowed to grow until tumors were established and had reached an approximate volume of 75 mm³. Concurrently, the DOPC liposomes and nanoparticles containing control or EphA2-targeting sequences were prepared and provided by the laboratories of Dr. Anil Sood and Dr. Gabriel Lopez-Berestein using siRNA sequences purchased from Sigma Genosys. siRNA known and shown to target EphA2 mRNA (5'-UGACAUGCCGAUCUACAUG-3') and siRNA known and shown to have no human mRNA target (5'-UUCUCCGAACGUGUCACGU-3') were used for liposomal and nanoparticle incorporation. Briefly, incorporation of DOPC and siRNA involved mixing the two in the presence of tertiary butanol, then Tween 20 was added, and finally an acetone/dry ice bath was used to freeze the mixture before being lyophilized (95). Preparation of the siRNA and nanoparticles involved the addition of TPP (thiamine pyrophosphate) and siRNA to a chitosan nanoparticle solution where the siRNA chitosan nanoparticles spontaneously formed (96). PBS, DOPC liposomes, or chitosan nanoparticles were injected i.v. twice weekly (150 µg/kg) for four weeks. Those groups that also received chemotherapy were injected i.p. with paclitaxel (24 mg/kg) once weekly in a PBS-glucose solution for four weeks. At the end of the

study, tumors were weighed and tumor tissue was collected for frozen sections, paraffin sections, and it was snap frozen for protein isolation.

Homogenization of Tumor Tissue for Protein Analysis

During mammary fat pad tumor collection, some tissue was snap frozen in liquid nitrogen for later protein analysis. Samples were homogenized while still frozen in TNE lysis buffer that consists of 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4 , 1 mM PMSF, and an inhibitor cocktail tablet. After homogenization, NP-40 (Sigma, Cat#N-6507) was added to each sample (1%v/v) and mixed well. Samples were incubated on ice for 30 min-1 hr. Homogenized tissues were then centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was removed and transferred to a clean microcentrifuge tube where it was centrifuged again. This process continued until a clear lysate was obtained. Protein concentrations could then be measured and were subjected to western blot analysis.

Immunohistochemistry for CD31 on Frozen Sections

Slides were fixed in acetone, washed, and incubated in 3% H_2O_2 in methanol. Sections were then protein blocked and the primary antibody (BD Pharmingen, Cat#553370) was added 1:1000 in protein block overnight at 4°C. The next day, slides were washed and the HRP secondary antibody (Jackson ImmunoResearch, Cat#112-035-167) was added 1:200 in blocking solution for 1hr at room temperature. Slides were washed, diaminobenzidine (DAB; Research Genetics, Cat#) was added, and then rinsed. Sections were

counterstained with Gill's Hematoxylin and rinsed. Finally, sections were allowed to dry and were mounted with Universal Mount.

Immunohistochemistry on Paraffin Embedded Tissue

For Ki-67 staining, slides were dewaxed. Antigen retrieval in citrate buffer (0.1M) was carried out. Slides were washed, incubated in endogenous peroxide blocking solution (3% hydroxyperoxide), and washed again. Protein blocking solution (TBST + 5%NGS) was applied to the slides for 1hr at room temperature. Primary antibody (Epitomics, Cat#4203-1) was added 1:200 in blocking solution and incubated at 4°C overnight. Slides were washed and secondary antibody (Jackson ImmunoResearch, Cat#111-036-047) was applied to sections 1:250 in blocking buffer and incubated for 1hr at room temperature. Slides were washed and incubated in DAB. Slides were washed, stained with hematoxylin, washed again, and then left to air dry before being mounted in Permount. For cleaved caspase 3 staining, slides were processed (de-waxed, antigen retrieval, and blocked for endogenous peroxidase) as described above. Antibody to cleaved caspase 3 (Cell Signaling, Cat#9661) was diluted 1:200 in protein blocking solution and incubated overnight at 4°C. The secondary antibody, DAB, and hemotoxylin counterstain was as described above.

Densitometry

The Image J program was downloaded and used to quantify protein levels revealed by western blot analysis. Each protein band was compared to its corresponding actin band by calculating the ratio. This ratio could then be compared with the other sample ratios.

Statistical Analysis

The results of the *in vitro* and *in vivo* studies were analyzed for their significance using the Student's T-test. A p-value of less than or equal to 0.05 was considered statistically significant. Calculations were carried out using Microsoft Office's Excel application.

- Chapter 3 -
Specific Aim I: Utilization of Targeted shRNA to
Study the Effects of Stable EphA2 Reduction *In*
vitro* and *In vivo

Specific Aim I: Utilization of Targeted shRNA to Study the Effects of Stable EphA2 Reduction *In vitro* and *In vivo*

Introduction

Stably reducing the levels of EphA2 in breast cancer cells was necessary in order to demonstrate that cells with low EphA2 expression would have decreased metastatic potential. Introducing synthetic siRNA into cells and allowing the natural cellular RNA interference process to use these siRNAs to silence protein expression is a powerful way to study the effects of EphA2 knockdown in metastatic breast cancer cell lines and in mouse xenograft models (97). However, a drawback of this method of RNAi pathway activation is that the effect of receptor expression silencing is transient, lasting only a few days (97). An alternative method of RNAi pathway activation can be mediated through the use of shRNAs (97). Plasmid vectors containing shRNA for the gene of interest can be inserted into a packaging cell lines which also contains plasmids encoding lentiviral proteins (97, 98). The gene of interest is then packaged into the virus which can be used to transduce a particular cell line where it will be randomly inserted into the host cell genome and will ultimately be incorporated into the RNAi pathway (97, 98).

Direct cellular delivery of siRNA, subsequent processing by the enzyme Dicer, and incorporation into RISC occurs in the cytoplasm of the cell (97, 98). While shRNA processing also occurs in the cytoplasm by Dicer, it is first processed in the nucleus by an enzyme called Drosha before cytosolic exportation (97, 98). This second method of RNAi pathway activation offers the advantage of longer-term silencing (98). The significance of this long-term silencing is that it allows investigation into the effects of

EphA2 receptor depletion over a period of time when the cellular mechanics are stably altered (98). The mechanism of using lentiviruses containing EphA2 targeting sequences for stable receptor depletion was adopted in order to conduct *in vitro* and *in vivo* studies.

Three EphA2-targeting sequences were separately inserted into lentivirus vectors in an attempt to achieve a stable knockdown of EphA2 receptor levels. These three viruses are termed LV3, LV4, and LV5. Additionally, there is control lentivirus that contains a scrambled, non-targeting sequence and it is termed LVNC. These virus names were used throughout the experiments to designate the cells that were transduced with a specific lentivirus. For example, MDA-MB-231 Luc cells transduced with LV3 were then termed MDA-MB-231 Luc LV3.

Results: *In vitro* Studies

Demonstration of Stable EphA2 Knockdown in MDA-MB-231 Luc, HCC1954, and BT549 Breast Cancer Cell Lines after Transduction with Lentivirus Containing EphA2-Targeting Constructs

Lentiviruses were designed that contained EphA2-targeting constructs for the purpose of stable protein knockdown. One (LV3) of the three (LV3, LV4, LV5) viruses created, each containing a unique targeting sequence, turned out to effectively reduce EphA2 receptor levels stably compared to the control (LVNC) that contains a non-targeting sequence. After transduction, cells were sorted based on GFP expression which indicates successful uptake and integration of viral information into the target cells. BT549 and MDA-MB-231 Luc cells transduced with LV3 showed the most significant protein reduction as revealed by western blot analysis and subsequent densitometry analysis. Further qPCR analysis confirmed the knockdown of EphA2 in the LV3 cell lines (data not shown) (Figure 6).

Figure 6. Demonstration of EphA2 Knockdown in MDA-MB-231 Luc, HCC1954, and BT549 Cell Lines after Transduction with Lentivirus

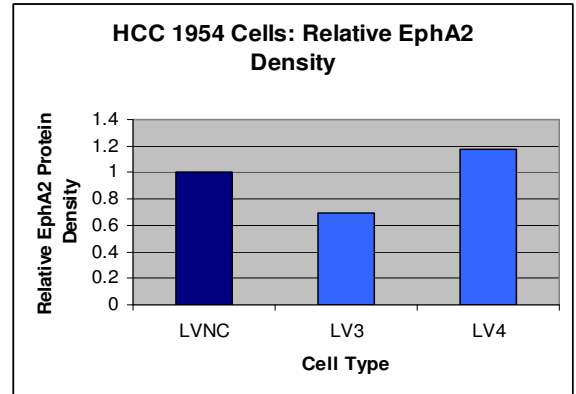
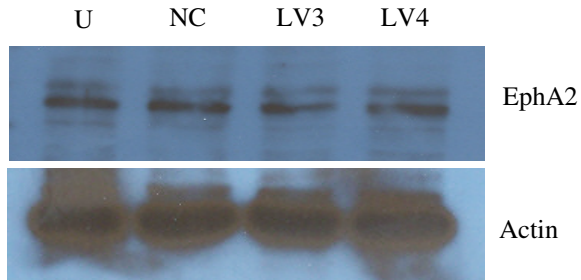
(A) Western blot analysis of protein lysates collected from HCC1954 cells that had been left untreated, treated with lentivirus containing a non-targeting construct, or treated with one of two lentiviruses containing EphA2-targeting constructs revealed that LV3 was most successful at reducing EphA2 protein levels (left). Actin was used as a control to ensure equal protein loading. Densitometry analysis confirmed the results of the western blot (right). LV3 was not as successful at stably reducing EphA2 protein levels in this cell line in comparison to the other two cell lines. **(B)** Western blot analysis of protein lysates collected from MDA-MB-231 Luc cells that had been left untreated, treated with lentivirus containing a non-targeting construct, or treated with one of three lentiviruses containing EphA2-targeting constructs revealed that LV3 was most successful at reducing EphA2 protein levels (left). Actin was used as a control to ensure equal protein loading. Densitometry analysis confirmed the results of the western blot (right). **(C)** Western blot analysis of protein lysates collected from BT549 cells that had been left untreated, treated with lentivirus containing a non-targeting construct, or treated with one of three lentiviruses containing EphA2-targeting constructs revealed that LV3 was most successful at reducing EphA2 protein levels (left). Actin was used as a control to ensure equal protein loading. Densitometry analysis confirmed the results of the western blot (right).

Figure 6

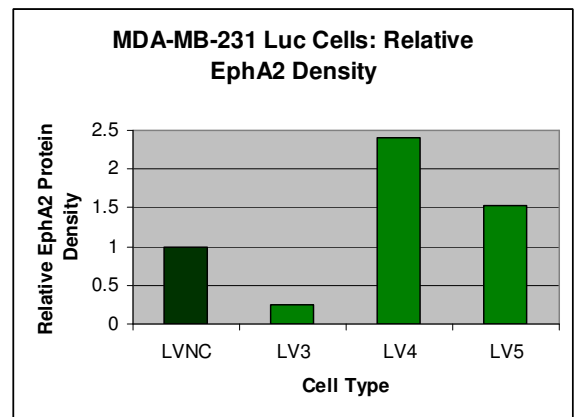
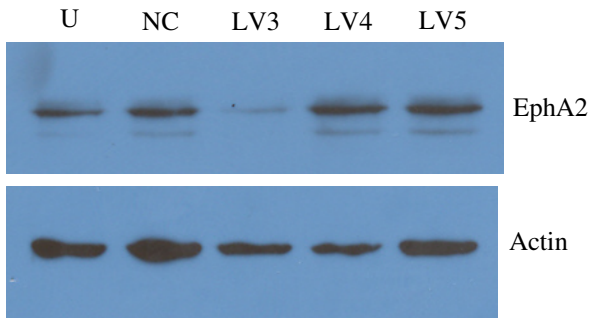
Demonstration of EphA2 Knockdown in MDA-MB-231 Luc, HCC1954, and BT549

Cell Lines after Transduction with Lentivirus

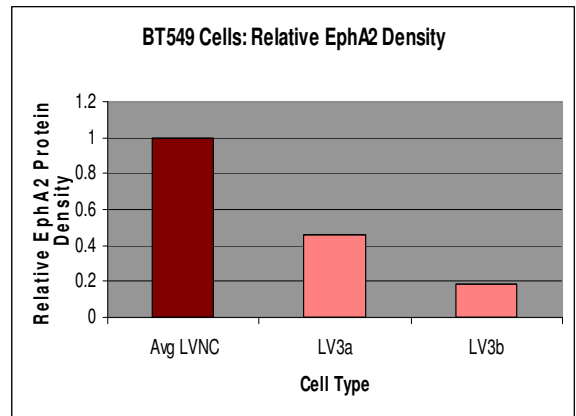
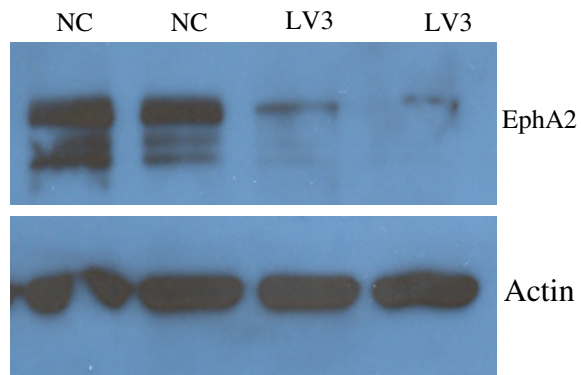
(A)



(B)



(C)



MDA-MB-231 Luc and BT549 Cells Transduced with LV3 Show Reduced Migration Towards the Chemoattractant EGF Compared to the Control

MDA-MB-231 Luc cells treated with lentivirus containing either the non-targeting control or EphA2-targeting construct were plated in a transwell chamber and stimulated by either 0.1%BSA or 50ng/ml EGF. MDA-MB-231 Luc LV3 cell migration was attenuated when stimulated by EGF in comparison to the 231 Luc LVNC cells where the number of migrated cells towards EGF was greater than towards 0.1%BSA. This result was significant when the number of MDA-MB-231 Luc LV3 cells that had migrated towards EGF was compared to the MDA-MB-231 Luc LVNC cells that had migrated towards EGF (Figure 7a). This migration experiment was repeated in the BT549 cell lines that had been transduced either by the EphA2-targeting lentivirus or the non-targeting lentivirus. The result was similar to that seen with the MDA-MB-231 Luc cells where the BT459 LV3 cells showed attenuated migration when compared to the BT549 LVNC cells (Figure 7b).

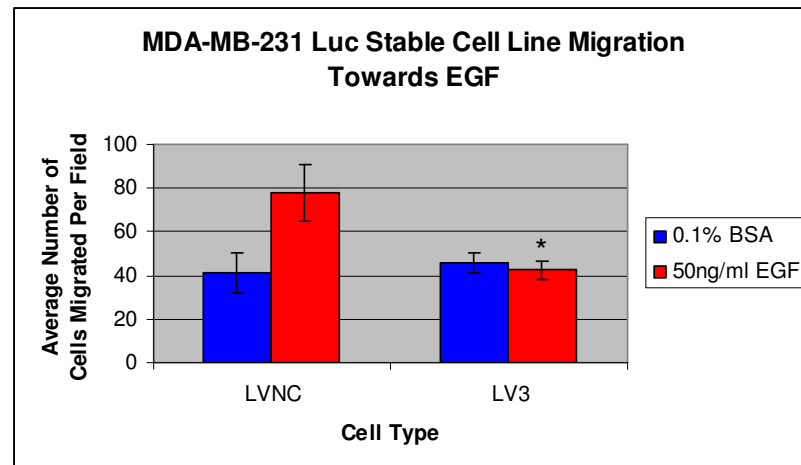
Figure 7. Cells Transduced with LV3 Showed Attenuated Migration Towards EGF

(A) MDA-MB-231 Luc LV3 and LVNC cells were plated in the upper chamber of a BioCoat transwell and stimulated with either 0.1% BSA or 50µg/ml EGF. The LV3 cells which have stably reduced EphA2 receptor levels showed attenuated migration in response to EGF when compared to the migration exhibited by the LVNC cells in response to EGF. This result is statistically significant when analyzed using the Student's T-test (p-value = 0.001). (B) BT549 LV3 and LVNC cells were plated in the upper chamber of a BioCoat transwell and stimulated with either 0.1% BSA or 50µg/ml EGF. The LV3 cells which have stably reduced EphA2 receptor levels showed attenuated migration in response to EGF when compared to the migration exhibited by the LVNC cells in response to EGF, but this finding was not significant (p-value = 0.9).

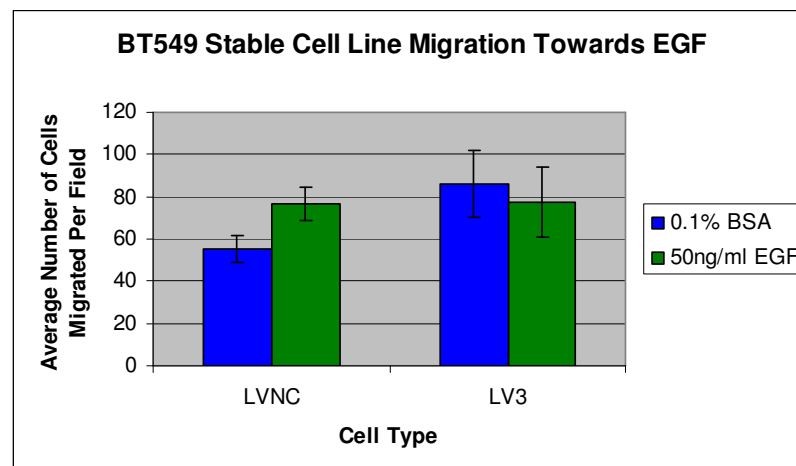
Figure 7

Cells Transduced with LV3 Showed Attenuated Migration Towards EGF

(A)



(B)



MDA-MB-231 Luc cells with Stably Reduced EphA2 Levels Show No Change in Anchorage Independent Growth Capability

The ability of cells to grow and colonize in soft agar indicates that they are malignant (99). Since EphA2 has been found to play a role in other hallmark processes of cancer such as migration and angiogenesis, it was thought that maybe it plays a role in anchorage independent proliferation and growth as well. 231 Luc, 231 Luc LVNC, and 231 Luc LV3 cells were suspended in soft agar containing either 1%FBS or 10%FBS. At the end of two weeks, the colonies that had formed were counted. There was no significant difference between the cell lines, revealing that reducing the receptor levels of EphA2 does not affect the anchorage independent growth characteristic of these breast cancer tumor cells (Figure 8).

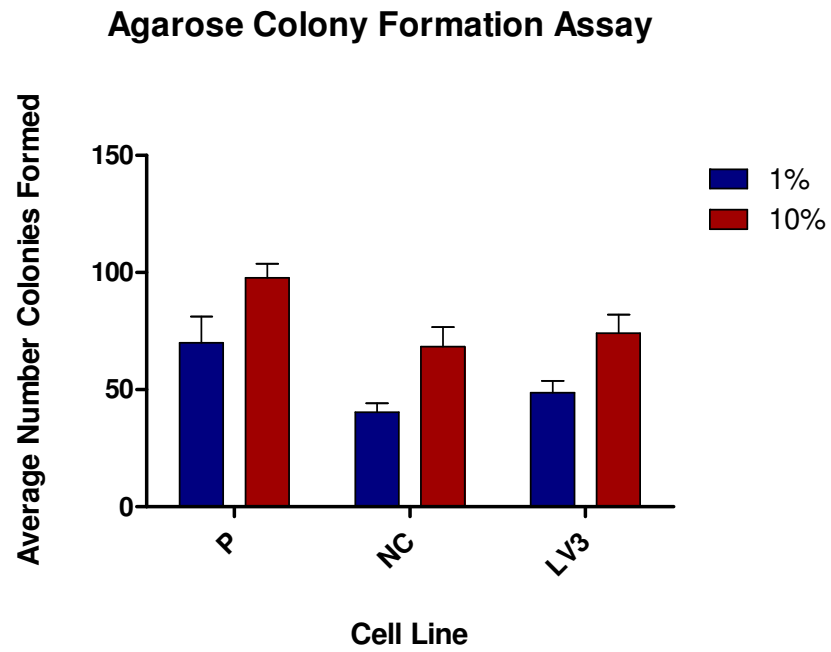
Figure 8. Reduced Levels of EphA2 Do Not Affect Anchorage Independent Growth

(A) MDA-MB-231 Luc, MDA-MB-231 Luc LVNC, and MDA-MB-231 Luc LV3 cells were plated in either 10% FBS or 1% FBS agarose solution and allowed to grow for 14 days. All cell lines showed a reduced number of cell colonies formed per field when plated in 1% FBS agarose solution and showed an increase in the number of cell colonies formed per field when plated in the agarose solution containing 10% FBS. The reduction in EphA2 receptor levels did not affect the ability of breast cancer cells to colonize independent of adherence and in reduced FBS conditions.

Figure 8

Reduced Levels of EphA2 Do Not Affect Anchorage Independent Growth

(A)



Results: *In vivo* Studies

Mammary Fat Pad Tumor Growth was Reduced in Mice Injected with the Stable MDA-MB-231 Luc LV3 Cell Line

Female athymic nude mice were injected in the mammary fat pad with either MDA-MB-231 Luc LVNC or MDA-MB-231 Luc LV3 cells to observe any differences in the tumorigenic ability between the two cell lines. Ten mice were injected with the LVNC cell line and ten mice were injected with the LV3 cell line. Tumor measurements were recorded and tumor volumes were calculated. Averages of the tumor volumes for each time point in each group were compared. Over the course of five and a half weeks, the mammary fat pad tumors of mice injected with MDA-MB-231 Luc LV3 cells showed a decrease in growth compared to the control group (Figure 9a). This indicates that EphA2 plays a role in tumorigenicity *in vivo*. Furthermore, analysis of the tumor weights of the LVNC and LV3 MFP tumors revealed a decrease in the average weight of the LV3 MFP tumors when compared with the LVNC average tumor weight (Figure 9b).

**Figure 9. Mammary Fat Pad Tumors with Stably Reduced EphA2 Showed
Decreased Tumor Growth**

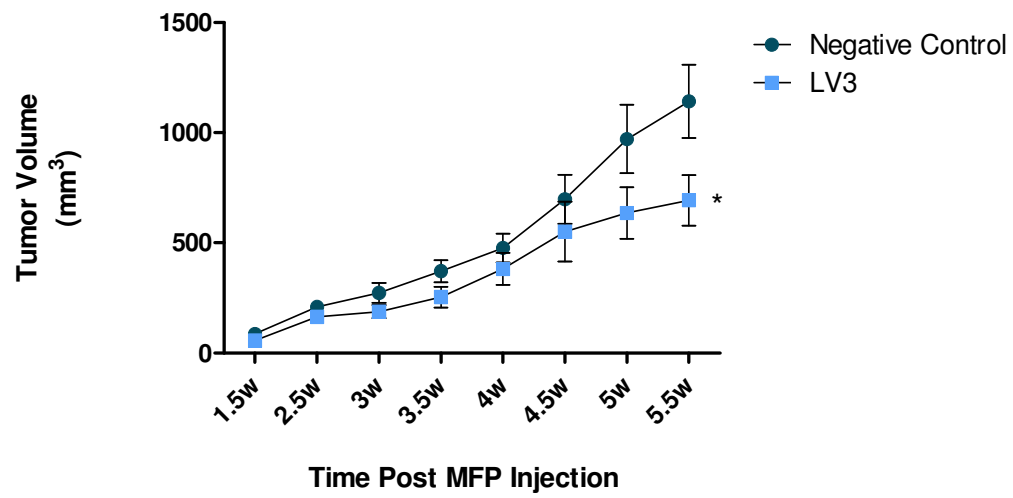
MDA-MB-231 Luc LVNC and MDA-MB-231 Luc LV3 cells were injected into the mammary fat pads of female athymic nude mice. After the tumors were established, the tumor dimensions were recorded and volumes were calculated. **(A)** Over the course of 5.5 weeks, the mammary fat pad tumors of the mice injected with the LV3 cells showed reduced tumor growth compared to the mammary fat pad tumors of those mice injected with the LVNC cells. This difference was significant as determined by the Student's T-test (p -value = 0.04) and was reproduced after a repeat experiment was conducted. **(B)** At the time of mice sacrifice, the MFP tumors were collected and weighed. Average tumor weight of the LVNC group is higher compared to the LV3 group. This data was not statistically significant (p -value = 0.347).

Figure 9

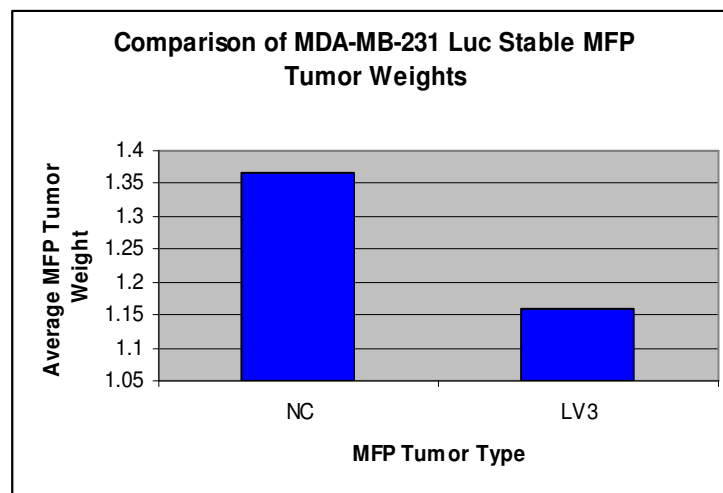
Mammary Fat Pad Tumors with Stably Reduced EphA2 Showed Decreased Tumor Growth

(A)

Growth Curve: Stable 231 Luc Cell Line MPF Injection



(B)



Tumor Protein Analysis Revealed Decreased EphA2 Protein Levels in the Mammary Fat Pad Tumors of Mice Injected with MDA-MB-231 Luc LV3 Cells

Having seen a reduction in mammary fat pad tumor growth in those mice that were injected with the MDA-MB-231 Luc LV3 cell line, the next step was to analyze the protein content of the tumor tissue to assess whether the decrease in tumor growth correlated with a reduction in tumor EphA2 protein levels. Western blot analysis revealed that EphA2 is reduced in the MDA-MB-231 Luc LV3 mammary fat pad tumors compared to the MDA-MB-231 Luc LVNC mammary fat pad tumors (Figure 10a). Further densitometry analysis confirmed a reduction in EphA2 levels in the MDA-MB-231 Luc LV3 mammary fat pad tumors relative to the average EphA2 content found in the control tumors (Figure 10b). Since a reduction in EphA2 led to a reduction in EGFR in the transient knockdown *in vitro* studies, the stable mammary fat pad tumor tissue protein lysates were also analyzed for EGFR protein levels (Figure 10a,c). It appears that there is a trend in EphA2 and EGFR protein levels in the stable mammary fat pad tumors where a reduction in EGFR correlated with the decrease in EphA2 protein levels. This trend was confirmed by densitometry analysis. While these data indicates a correlation in EphA2 protein levels and tumor growth, it was not found to be statistically significant.

**Figure 10. EphA2 Protein Levels Were Decreased in Mammary Fat Pad Tumors
with Stably Reduced EphA2**

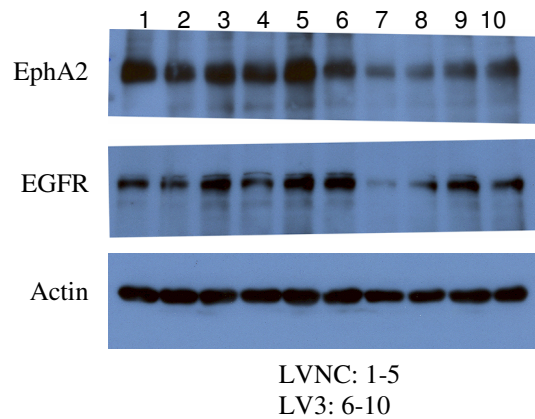
(A) Protein was isolated from MDA-MB-231 Luc LVNC and MDA-MB-231 Luc LV3 mammary fat pad tumor tissue and subjected to western blot analysis to reveal EphA2, EGFR, and actin protein levels. Actin was blotted for to ensure that equal amounts of protein were loaded. 20 μ g of protein was loaded into each lane. Lanes 1-5 represent five distinct MDA-MB-231 Luc LVNC mammary fat pad tumors and lanes 6-10 represent five distinct MDA-MB-231 Luc LV3 mammary fat pad tumors. (B) Densitometry analysis confirmed the data obtained from the western blot analyses. The individual EphA2 protein densities of the MDA-MB-231 Luc LVNC mammary fat pad tumors were compared to their respective actin densities (EphA2/actin) and then all of the individual LVNC protein densities were averaged and the average was divided by itself to obtain a reference point of 1. The individual EphA2 protein densities of the MDA-MB-231 Luc LV3 mammary fat pad tumors were compared to their respective actin densities (EphA2/actin) and then divided by the average of the LVNC densities to show the amount of EphA2 protein reduction in the individual LV3 mammary fat pad tumors relative to the negative control mammary fat pad tumors. (C) The same method of densitometry analysis described in (B) was applied to the EGFR protein densities to show a correlation in EphA2 reduction and EGFR reduction.

Figure 10

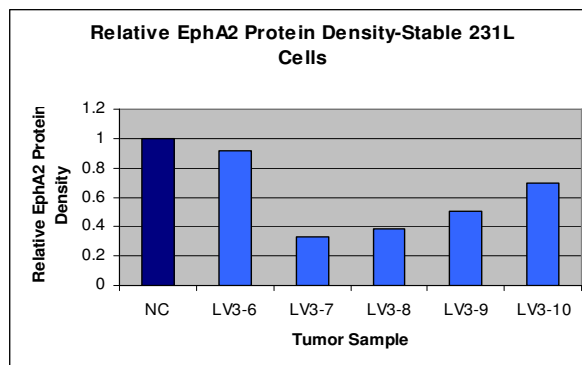
EphA2 Protein Levels Were Decreased in Mammary Fat Pad Tumors with Stably

Reduced EphA2

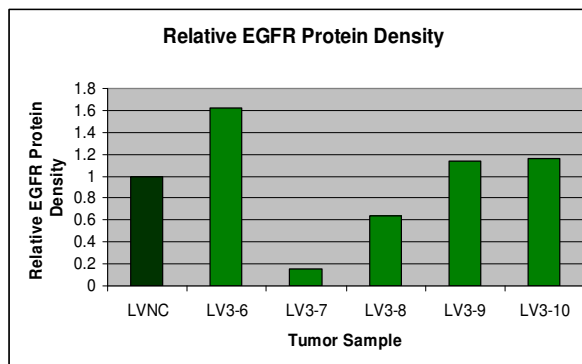
(A)



(B)



(C)



The MVD in MDA-MB-231 Luc LV3 Mammary Fat Pad Tumors was Reduced Compared to the MVD in MDA-MB-231 Luc LVNC Mammary Fat Pad Tumors

Previous tumor growth and tumor protein analysis indicated that the MDA-MB-231 Luc LV3 cells exhibited slower growth and also had reduced amounts of EphA2 protein when compared to the control tumors. Since angiogenesis, like proliferation, is a hallmark of cancer, it was hypothesized that reduced EphA2 would also affect vessel growth and formation in the MDA-MB-231 Luc LV3 tumors. CD31 staining was carried out on tissue sections prepared from MDA-MB-231 Luc LVNC and MDA-MB-231 Luc LV3 mammary fat pad tumors (Figure 11a,b). Tissue sections were observed under 20x magnification and vessel density was quantified by counting the number of individual vessels inside a 0.25 x 0.25mm grid. Tumor vessel density was modestly, but significantly reduced in the MDA-MB-231 Luc LV3 tissue sections when compared to the MDA-MB-231 Luc LVNC vessel density (Figure 11c). Staining for Ki-67 and cleaved caspase 3 was also done, although, this data was uninformative because it did not reveal a difference between the LVNC and LV3 tumor tissue samples (data not shown).

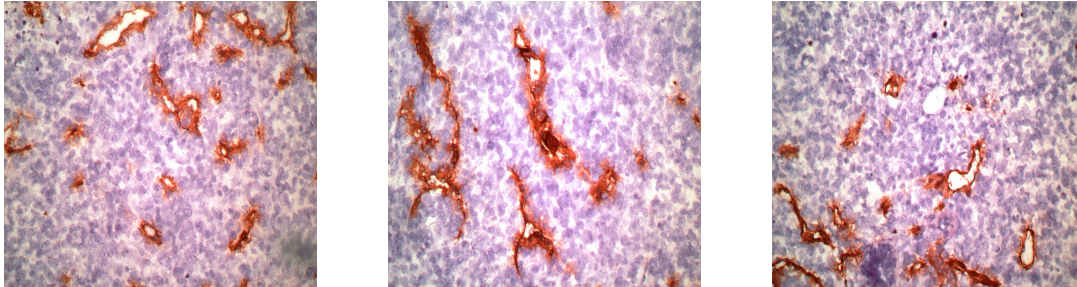
**Figure 11. Reduced MVD in MDA-MB-231 Luc LV3 Mammary Fat Pad Tumors
Compared to MDA-MB-231 Luc LVNC**

(A) CD31 stained MDA-MB-231 Luc LVNC tumor tissue sections revealed a high microvascular density and more vessels with visible lumens. (B) CD31 stained MDA-MB-231 Luc LVNC tumor tissue sections revealed a lower microvascular density with fewer vessels having visible lumens when compared to the LVNC stained tissue sections. (C) Vessel density was counted in ten different locations within one tumor section (each tumor section being from a different mouse mammary fat pad tumor) and then averaged to get a representative number of vessels per field. Quantification of the vessel density revealed a statistically significant (p -value = 0.002) reduction in the number of vessels per field in the CD31 stained MDA-MB-231 Luc LV3 tumor tissue sections.

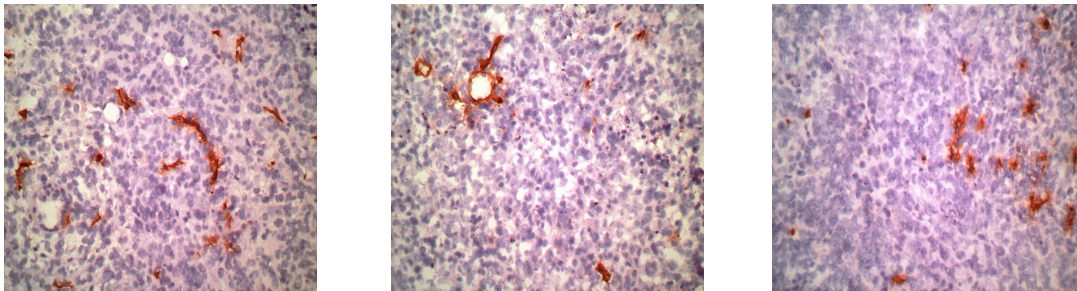
Figure 11

**Reduced MVD in MDA-MB-231 Luc LV3 Mammary Fat Pad Tumors Compared to
MDA-MB-231 Luc LVNC**

(A) MDA-MB-231 Luc LVNC CD31 Tumor Tissue Staining

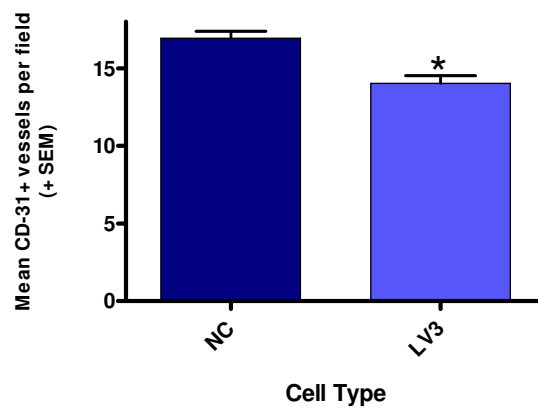


(B) MDA-MB-231 Luc LV3 CD31 Tumor Tissue Staining



(C)

**Quantification of MVD in MDA-MB-231 Luc
LVNC or LV3 MFP Tumors**



MDA-MB-231 Luc LV3 Cells Develop Fewer Experimental Lung Metastases in Comparison to LVNC Cells After Intravenous Injection into Mice

Athymic female nude mice were injected i.v. with either MDA-MB-231 Luc LVNC or MDA-MB-231 Luc LV3 cells in order to observe the efficiency of the two cell lines in developing experimental metastases. Intravenous injections allows for cell circulation and ultimate arrest in the capillary beds of various tissues. The development of lung metastases was measured using the IVIS (Caliper) after i.p. injection of luciferin.

Luciferin is oxidized in the presence of luciferase (expressed by the MDA-MB-231 Luc cells) and light energy is a by-product. This light energy, or luminescence, can be detected and quantified by the IVIS machine. Based on initial readings at 3 and 4 weeks after injection, more mice in the LVNC group developed lung metastases compared to the LV3 group (Figure 12a). This suggests that breast cancer cells with reduced EphA2 are not as efficient at developing lung metastases as the cells that over express EphA2.

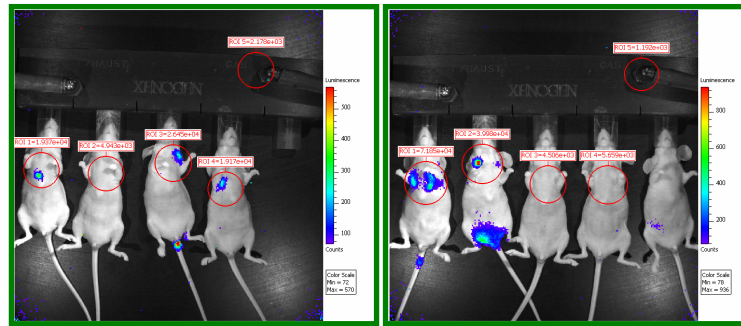
Figure 12. Breast Cancer Cells with Stably Reduced EphA2 Expression Do Not Develop Lung Metastases As Efficiently as Breast Cancer Cells Overexpressing EphA2

MDA-MB-231 Luc LVNC or LV3 cells were injected intravenously into athymic female nude mice. 1×10^6 cells suspended in 0.2ml PBS were injected in each mouse and there were 10 mice per cell line. (A) Four weeks post-i.v. injection, the mice were imaged after being injected i.p. with 0.2ml of luciferin (15mg/ml). Imaging of the LVNC mice revealed lung metastases in 5 of the 9 mice (the two upper images). This number had increased from imaging done the week before where only 2 of the 9 mice displayed lung metastases. In contrast, imaging of the LV3 mice revealed that only one of the ten mice had a lung metastasis and another mouse had a possible bone metastasis (the two upper lower images). There was no additional development of lung metastases or other bodily metastases in these two mice when compared to imaging done the previous week, and the additional mice in the LV3 group had still not developed metastases at all.

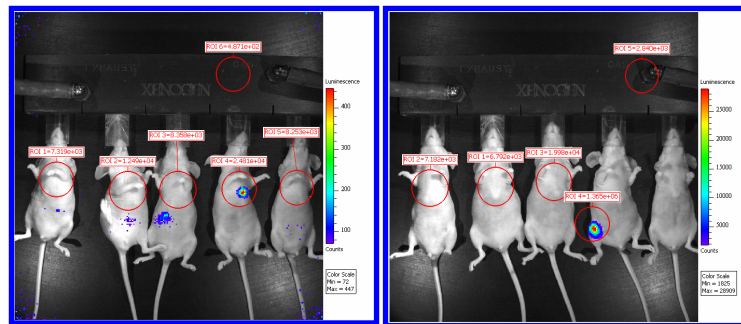
Figure 12

**Breast Cancer Cells with Stably Reduced EphA2 Expression Do Not Develop Lung
Metastases As Efficiently as Breast Cancer Cells Overexpressing EphA2**

(A) IVIS Imaging LVNC and LV3 Mice: 4 weeks After Injection



LVNC Mice



LV3 Mice

Discussion: Utilize Targeted shRNA to Study the Effects of Stable EphA2 Reduction *In vitro* and *In vivo*

The goal of Specific Aim I was to develop a stable knockdown of EphA2 in metastatic breast cancer cell lines to study the effect of receptor depletion over an extended period of time. Metastatic breast cancer cell lines with a stable knockdown of EphA2 were used to complete *in vitro* experiments for properties indicating metastatic potential that have helped to confirm the findings of the transient siRNA experiments, in which EphA2 was only maximally reduced for a few days. Use of these cell lines with stably depleted EphA2 expression also assisted in determining how EphA2 influences tumorigenicity *in vivo*, a process that cannot be successfully studied using transient siRNA-mediated knockdown. These *in vitro* and *in vivo* experiments have helped to reinforce the hypothesis that EphA2 contributes to the ability of breast cancer cells to metastasize.

First and foremost, it was imperative to find a way to stably reduce EphA2 receptor expression in metastatic breast cancer cell lines. The first attempt involved the transformation of competent bacterial cells by introducing EphA2 shRNA construct-containing plasmids to the cells and subsequently amplifying the cultures. In addition to containing an EphA2 shRNA construct, the plasmids also carried a puromycin resistance gene so that transfected cells could be selected for based on their resistance. Despite successful transfection of the cells with the EphA2 shRNA-containing plasmids (assuming puromycin resistance is indicative of successful cellular uptake of the plasmid), there was no reduction in EphA2 receptor levels, or if there was, it was too

minimal to expect significant results if used for *in vitro* and *in vivo* experiments. After several unsuccessful attempts to stably reduce EphA2 after plasmid transfection, a mixture of 3-5 lentiviral vector plasmids with shRNA sequences targeting EphA2 were purchased from Santa Cruz in hopes that this alternative method would prove the desired stable knockdown. However, these lentiviral particles were not successful either. Next, the EphA2 siRNA sequences that worked very well for transient knockdown were used to create shRNA sequences. After packing these sequences in lentiviruses, they were used to transfect cells. Again, these did not produce the desired stable EphA2 knockdown result. Ultimately, the custom made lentivirus #3 (described in the Materials and Methods) containing the EphA2 shRNA construct obtained using Invitrogen's RNAi Designer ended up being the only vector that led to a significant and stable depletion of EphA2 at both the protein and mRNA levels in two metastatic breast cancer cell lines (Figure 6). The point of describing the various attempts that were made to achieve a stable knockdown of EphA2 in metastatic breast cancer cell lines is to convey the message that the *in vitro* and *in vivo* results based on stable EphA2 receptor knockdown would have been strengthened if they could have been repeated using an additional shRNA construct, but this turned out to be unachievable with the numerous methods tested to date. Therefore, the experiments were carried out with the use of only one lentiviral vector plasmid containing an EphA2 encoding shRNA sequence, LV3.

EphA2 has been implicated to play a role in migration in the head and neck carcinoma cell line, HN5, and in the A431 cell line (100). Not only is EphA2 expression upregulated by EGF-activated EGFR, but treatment of the aforementioned cell lines with EphA2-targeting siRNA resulted in an inhibition of migration as determined by wound

healing assays (100). In addition, EGF has been shown to induce migration of MDA-MB-231 breast cancer cells, but this finding did not include EphA2 as a part of the mechanism. This EGF-induced migration of metastatic breast cancer cells was confirmed and expanded upon by the work done in this thesis in the MDA-MB-231 Luc and BT549 cells (Figure 7). The use of EphA2 siRNA in these migration experiments revealed that EphA2 is most likely a participant in this EGF-activated EGFR migratory pathway and other studies using alternative cancer models have shown this to be the case as well (100, 101). The hypothesis that EphA2 plays a role in the metastatic process by affecting the migratory ability of breast cancer cells was first confirmed by using EphA2 siRNA to reveal a reduction in EGF-induced migration. Lentiviral transduction of BT549 and MDA-MB-231 Luc cells with either the non-targeting or EphA2-targeting constructs was performed to show that the breast cancer cell lines with stably reduced EphA2 still showed a reduction in EGF-induced migration, thus confirming the results obtained with transient silencing of EphA2. The mechanism underlying this reduced migration is still being elucidated, but it has been demonstrated that activated EGFR phosphorylates Akt which serine phosphorylates EphA2, leading to the formation of a protein complex, subsequent activation of Rac1, and initiation of migration (102, 103).

To continue investigating the effects of stable EphA2 receptor knockdown, anchorage-independent growth assays were conducted to see if EphA2 might play a role in the ability of breast cancer cells to grow independent of attachment to plastic (Figure 8). Normal epithelial cells usually cannot survive detachment from the extracellular matrix (101, 104). Once detached, these cells undergo programmed cell death called anoikis (104). However, metastatic cancer cells have acquired the ability to overcome cell

death as a result of ECM detachment and can survive longer in suspension (104). This gives cancer cells the ability to survive after dissociation from the primary tumor where they now have a high likelihood of metastasizing (101, 104). Suspension of MDA-MB-231 Luc LVNC and MDA-MB-231 Luc LV3 cells in agar containing either 10% FBS or 1% FBS did not reveal any anchorage independent growth differences between the cell lines. This result suggests that EphA2 does not play a role in anchorage independent growth of these cells. One study suggests that EphA2 expression is restricted to adherent cells and is actually decreased upon detachment from plastic *in vitro* (101). Therefore, it is possible that stably reduced EphA2 expression does not affect the anchorage independent growth of metastatic breast cancer cells and the downregulation after cell detachment seen in this previous study may be an explanation as to why there is no difference between the MDA-MB-231 Luc LVNC and MDA-MB-231 Luc LV3 cell lines.

Another use for the stable knockdown cell lines is to elucidate the effect that EphA2 may have on tumorigenicity in the mammary fat pad of mice. Delivery of siRNA to mammary fat pad tumors is one strategy to study how receptor knockdown influences tumor growth and other properties after tumor establishment, but it cannot indicate the role that reduced EphA2 plays in the actual tumor establishment process. Athymic female nude mice that had been injected in the mammary fat pad with MDA-MB-231 Luc LV3 showed decreased tumor growth when compared to the mice injected with the MDA-MB-231 Luc LVNC cells (Figure 9). This experiment was repeated and the resultant data confirmed the findings these initial findings. It appears that EphA2 did affect the ability to form tumors in the mammary fat pad although it is not clear exactly what is happening

here. Protein analysis by western blot and densitometry confirmed that the LV3 mammary fat pad tumors had decreased EphA2 protein levels and had a slight reduction in EGFR protein levels as well (Figure 10). However, given that EphA2 does not play a well-characterized role in cell growth and proliferation in MDA-MB-231 cells (101), it is unknown exactly how proliferation is being affected. EphA2 may not be affecting tumor growth directly, however, given the established role of EGFR in promoting cancer cell proliferation, it is possible that the decrease in tumorigenicity is actually an indirect effect of EphA2 somehow reducing the EGFR levels in the tumor cells (101). However, staining for proliferation and apoptosis markers (Ki-67 and cleaved caspase-3) did not show any differences between MDA-MB-231 Luc LVNC tumor sections and MDA-MB-231 Luc LV3 tumor sections. These results could be due to the relatively small difference in tumor sizes between the LVNC and LV3 groups, or it could be due to inadequate sampling of slides. Staining tumor tissue sections for CD31 revealed a significant decrease in the density of microvessels in the LV3 tumors compared to the LVNC tumors (Figure 11), but *in vitro* studies assaying for VEGF concentrations in the EphA2 reduced cells did not reveal a difference in concentration compared to the control cells. However, other cytokines regulating angiogenesis may be altered. For example, it is possible that EphA2 receptor depletion may have more of an effect on matrix metalloproteinases, MMPs, which are also involved in the angiogenic process (105). The trend seen in decreased EphA2 and decreased microvascular density in tumor tissue remains unclear. A more comprehensive investigation, such as expression profiling of RNA from tumor tissues, might provide insight into why the tumors with lower EphA2 expression grew more slowly than the control counterparts.

Furthermore, stable knockdown of EphA2 was required to understand the role this receptor plays in the development of metastases *in vivo*. Intravenous injection of the MDA-MB-231 Luc LVNC or LV3 cells into mice revealed a correlation between reduced EphA2 and a decrease in the number of mice that developed lung metastases (Figure 12). This suggests that EphA2 plays a role in the ability of cancer cells to establish secondary tumors once they have dislodged from the primary tumor, entered circulation, and arrested in the capillary beds of other tissues within the body. Reducing the expression of EphA2 in EphA2-expressing breast carcinomas might be one way to reduce the incidence of metastasis.

The results of the *in vitro* and *in vivo* experiments involving MDA-MB-231 Luc and BT549 cells with stably reduced EphA2 clearly show that there is a trend between diminished receptor levels, decreased migration, decreased tumorigenicity, decreased vessel formation, and reduced development of lung metastases, although, the exact cellular mechanics behind these findings are still unclear. Further work needs to be done to strengthen the current results and to elucidate the pathways involved.

- Chapter 4 -
Specific Aim II: siRNA-Mediated Transient
Reduction of EphA2

Specific Aim II: Utilization of Targeted siRNA to Study the Effect of Transient EphA2 Reduction *In vitro* and *In vivo*

Introduction

Since its discovery, EphA2 has been found to be upregulated during specific developmental processes and also during the process of mammary branching morphogenesis in the pubescent female breast (43, 47, 48, 51). However, aside from these two key periods of upregulation, EphA2 expression is low and limited to epithelial tissues (53, 54). Interestingly, this normal pattern of EphA2 expression is disrupted during the development of aggressive carcinomas such as those of the prostate, pancreas, and ovaries, where EphA2 is aberrantly regulated which results in receptor overexpression (63, 65, 66). Similar to the aforementioned types of cancer, EphA2 overexpression is also consistently found in the more aggressive and metastatic breast cancers (77). Investigation into the role of EphA2 in the process of malignant transformation of normal breast epithelium has identified it as an oncoprotein whose overexpression can single-handedly promote normal cell transformation to a cancerous and subsequently metastatic state (50, 53). Furthermore, molecules whose aberrant regulation or expression is classically associated with cancer promotion have been found to regulate EphA2. Such molecules include the estrogen hormone, E-cadherin, and LMW-PTP. Indeed, dysregulation of these molecules contributes to the overexpression and cancer-promoting functions of EphA2 (53, 59, 75, 76, 84).

The overexpression of EphA2 exhibited in aggressive forms of various cancers with high metastatic potential has made it the focus of investigations aiming to define and

target novel molecules that have potential therapeutic significance (106). Small-interfering ribonucleic acid (siRNA) that targets EphA2 mRNA to transiently reduce protein levels was shown to attenuate pancreatic tumor growth in xenograft models (64). Furthermore, EphA2-targeting siRNA delivered in 1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) neutral liposomes had a similar negative affect on tumor growth in ovarian cancer studies (95, 107). In breast cancer, adenoviral vectors constructed to express the EphA2 ligand, ephrinA1, were introduced to breast cancer cell lines and demonstrated reduced tumorigenic potential after EphA2 receptor depletion (62). In addition, ectopic overexpression of EphA2 could be reversed by using an EphA2-targeting antibody, which resulted in an increase in sensitivity to chemotherapeutic agents (108). However, while various methods have been utilized in breast cancer studies to reduce EphA2 levels, the siRNA transient silencing approach and its ability to affect indicators of tumorigenic and metastatic potential *in vitro* and *in vivo* hasn't been extensively studied.

The goal of Specific Aim II is to elucidate the affect of EphA2-targeting siRNA *in vitro* and *in vivo* with the intention of more fully understanding the biology behind this receptor's mechanism of action and possibly identifying a novel therapy for the treatment of metastatic breast cancer. A variety of breast cancer cell lines were analyzed for EphA2 protein expression levels and a few specific, overexpressing lines were chosen for use in this study. Initial experiments revealed the efficacy of different EphA2-targeting siRNA's in successfully reducing receptor protein levels. The optimal siRNA constructs were then used to treat cells prior to conducting various *in vitro* experiments such as migration assays, proliferation assays, and growth factor release assays, all of which

would aid in determining the effect of EphA2 protein reduction on metastatic potential. *In vivo* studies were carried out by monitoring the effect of treating established mammary fat pad tumors with EphA2-targeting siRNA delivered in either DOPC liposomes or attached to chitosan nanoparticles. Tumor volume and weights were recorded and post-treatment analysis of tumor tissue included an evaluation of EphA2 protein levels, tumor cell proliferation, and microvascular density.

Results: *In vitro* Studies

Demonstration of EphA2 Expression at the Protein and mRNA Levels in a Variety of Breast Cancer Cell Lines

Protein isolated from a panel of breast cancer cell lines, with varying degrees of aggressiveness and metastatic potential, was subjected to western blot analysis to reveal differences in the levels of EphA2 protein expression within the various lines (Figure 13a). Furthermore, RNA isolated from this same panel of breast cancer cell lines was analyzed by qPCR to quantify levels of EphA2 mRNA (Figure 13b). The breast cancer cell lines with the highest EphA2 protein levels had correlating mRNA levels, and according to the breast cancer subtype classification system proposed by Neve et al, the majority of those cell lines with robust EphA2 expression are also of the basal subtype (8). This confirms and establishes reports that have found a correlation between ER-negative, basal subtype breast cancers and EphA2 overexpression (7, 26, 33, 75). As previously mentioned, the majority of breast cancer-related deaths result from the disease developing metastatic capabilities and causing complications at distant, secondary sites (3). Based on the expression data collected and the knowledge that metastasis is a serious clinical challenge, the MDA-MB-231, HCC1954, and BT549 cell lines were chosen to be the chief cell lines used in my studies due to their relative aggressiveness and high EphA2 expression (89).

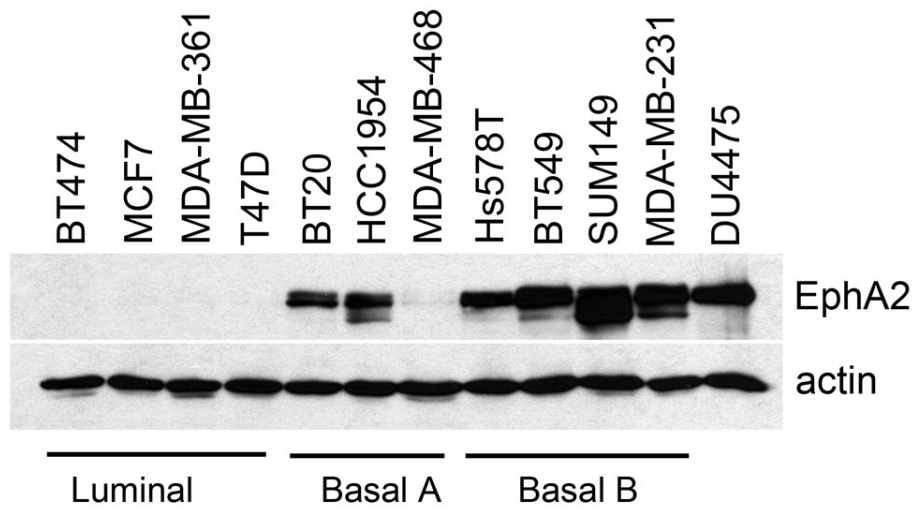
Figure 13. EphA2 Protein and mRNA Expression Levels

Western blot analysis of protein lysates collected from twelve breast cancer cell lines and probed with an anti-Eck/EphA2 monoclonal antibody. The separated protein lysates were also probed with an anti-actin antibody to ensure equal protein loading. High EphA2 protein levels were shown to be expressed by a majority of the basal subtype breast cancer cell lines. This unpublished data was provided by a previous member of the lab. **(B)** Total RNA was extracted from the same twelve breast cancer cell lines and used to carry out quantitative PCR analysis. EphA2 mRNA levels were elevated in the same cell lines that had elevated EphA2 protein levels.

Figure 13

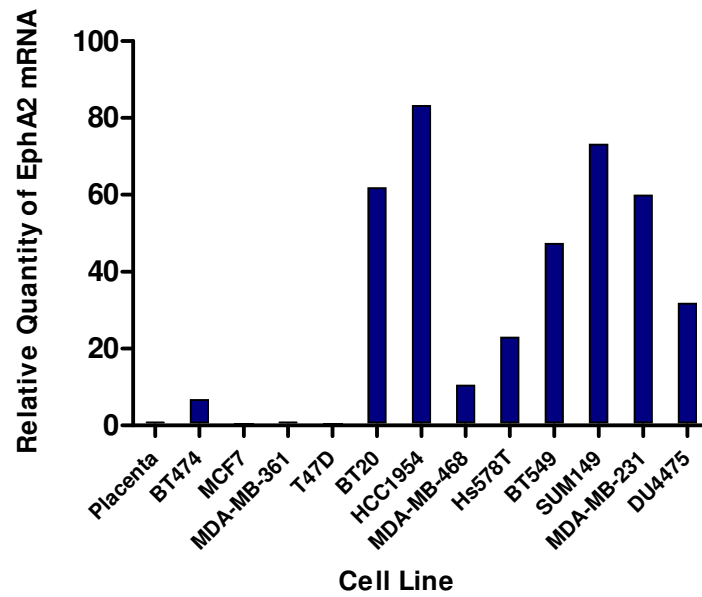
EphA2 Protein and mRNA Expression Levels

(A)



(B)

Relative Quantities of EphA2 mRNA



Use of EphA2-Targeting siRNA to Transiently Reduce Protein Levels in the MDA-MB-231, HCC1954, and BT549 Cell Lines

Small-interfering RNA's are double-stranded RNA's that are normally 20-25 nucleotides in length. The two strands are hybridized in such a way that there is a single stranded over-hang at either 3' end. Transfection reagents make the cell membrane permeable so that siRNA can enter. Once inside the cell, the siRNA is incorporated into a complex called a RNA-Induced Silencing Complex (RISC) that has endonuclease activity. The siRNA is unwound and directs the entire complex to a complementary messenger RNA (mRNA) to which the complex binds, cleaves, and subsequently destroys (109, 110).

Two siRNA constructs purchased from Ambion (Applied Biosystems) that target EphA2 mRNA were shown to work effectively in various cell lines to transiently reduce EphA2 protein levels. These two siRNA constructs were used to treat the MDA-MB-231 Luc (Figure 14a), HCC1954 (Figure 14b), and BT549 (Figure 14c) cell lines to achieve successful transient knockdown. MDA-MB-231 Luc cells treated with the targeting siRNA's exhibited an almost complete knockdown of EphA2 protein, with HCC1954 and BT549 cells achieving similarly high levels of protein reduction.

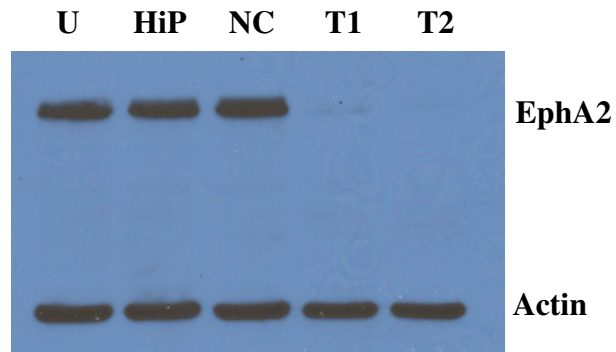
Figure 14. siRNA-Mediated Transient Reduction of EphA2 Protein Levels

Lanes are designated as one of the following: **U.** Untreated cells **HiP.** Hiperfect transfection reagent added to cells **NC.** Non-targeting siRNA construct added to cells in addition to Hiperfect transfection reagent **T1.** Cells treated with EphA2-targeting siRNA #1 (146) plus Hiperfect transfection reagent **T2.** Cells treated with EphA2-targeting siRNA #2 (242) plus Hiperfect transfection reagent. **(A)** MDA-MB-231 Luc cells revealed successful EphA2 transient knockdown after treatment with the targeting siRNA constructs in comparison to treatment with the non-targeting siRNA construct. **(B)** HCC 1954 cells revealed successful EphA2 transient knockdown after treatment with the targeting siRNA constructs in comparison to treatment with the non-targeting siRNA construct. **(C)** BT549 cells revealed successful EphA2 transient knockdown after treatment with the targeting siRNA constructs in comparison to treatment with the non-targeting siRNA construct.

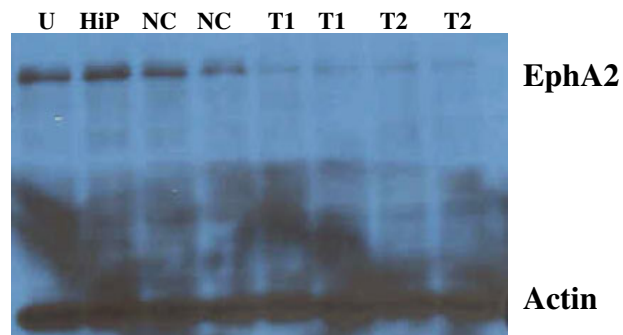
Figure 14

siRNA-Mediated Transient Reduction of EphA2 Protein Levels

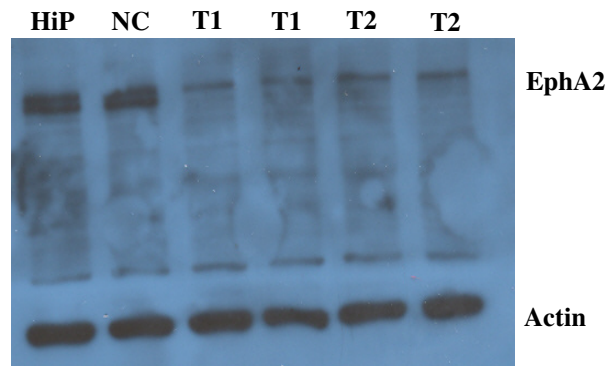
(A) MDA-MB-231 Luc



(B) HCC1954



(C) BT549



Cells with Transiently Reduced EphA2 Protein Levels Exhibited a Decrease in Migratory Ability Towards Chemoattractants

The migratory ability of breast cancer cells is important because it allows for progression to an invasive and metastatic state (111). In response to 50ng/ml EGF, MDA-MB-231 Luc and BT549 cells left untreated, treated with the Hiperfect transfection reagent alone, or treated with non-targeting siRNA showed greater migration than those cells with transiently reduced EphA2 (Figure 15a,b). HCC1954 cells showed modest differences in migration when comparing control cells with EphA2-reduced cells. This subtleness could be due to the amplification of HER2/neu which could work through other pathways to stimulate migration, overcoming the effect of EphA2 depletion on migration towards EGF in this cell line (results not shown).

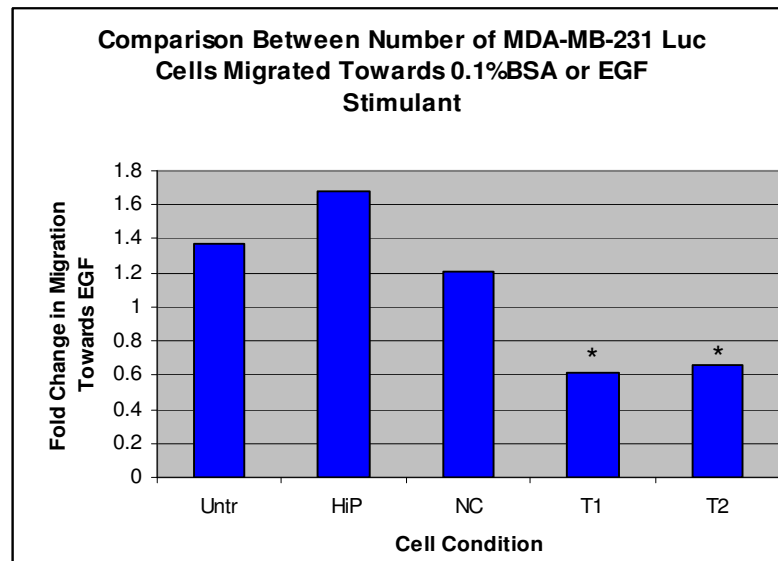
**Figure 15. Transient EphA2 Reduction Attenuates Migration Towards
Chemoattractants**

(A) MDA-MB231 Luc cells were either left untreated, treated with the Hiperfect transfection reagent alone, treated with non-targeting siRNA, or treated with one of two EphA2-targeting siRNA. Cells were then collected, suspended in serum-free media, and placed in the upper portion of a transwell chamber. The lower portion contained either 0.1% BSA in medium or 50ng/ml EGF in medium. Cells were incubated and allowed to migrate for 6 hours after which they were fixed, stained, and migrated cells were quantified. Cells that had been treated with the EphA2-targeting siRNA showed attenuated migration in comparison to the various controls. Application of the Student's t-test found this data to be statistically significant when comparing migration of the knockdown cell lines towards EGF and the negative control cell line towards EGF. The p-value was 0.02 for T1 and 0.002 for T2. (B) BT549 cells were either left untreated, treated with the Hiperfect transfection reagent alone, treated with non-targeting siRNA, or treated with one of two EphA2-targeting siRNA. Cells were then collected, suspended in serum-free media, and placed in the upper portion of a transwell chamber. The lower portion contained either 0.1% BSA in medium or 50ng/ml EGF in medium. Cells were incubated and allowed to migrate for 6 hours after which they were fixed, stained, and migrated cells were quantified. Cells that had been treated with the EphA2-targeting siRNA showed attenuated migration in comparison to the various controls. Application of the Student's t-test found this data to be statistically significant when comparing migration of the knockdown cell lines towards EGF and the negative control cell line towards EGF. The p-value was 0.0004 for T1 and 0.0002 for T2.

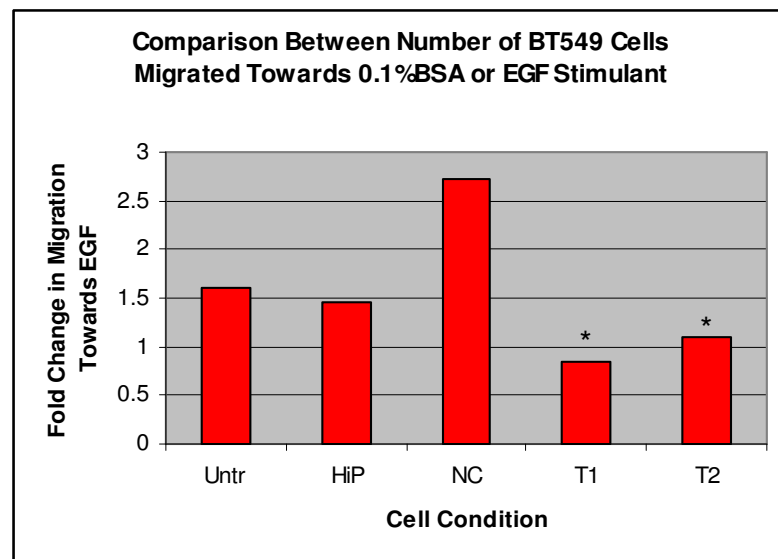
Figure 15

Transient EphA2 Reduction Attenuates Migration Towards Chemoattractant

(A)



(B)



Investigation into the Relationship between EphA2 and EGFR in Promotion of Cancer Cell Migration

The EphA2-EGFR relationship has been explored in other cancer cell lines. EGFR activation by its ligand was shown to induce EphA2 expression in the human head and neck carcinoma cell line, HN5, and in a cell line derived from a human epidermoid carcinoma in the vulva, A431 (100). Further studies have revealed that EphA2 and EGFR colocalize on the plasma membrane of these cells and can be co-immunoprecipitated, providing evidence that these two receptors physically interact (100). Furthermore, EphA2 knockdown reduced cell migration towards EGF, suggesting that the EphA2 receptor plays a role in EGF-stimulated motility in these cells (100). We sought to find out if the same relationship existed in breast cancer cell lines since EphA2 depletion also leads to reduced EGF-stimulated migration in our cells. Western blot analysis revealed that siRNA-mediated transient reduction of EphA2 in the MDA-MB-231 Luc and BT20 breast cancer cell lines led to reduced EGFR receptor expression (Figure 16a). Total RNA was extracted from the same twelve breast cancer cell lines used to analyze EphA2 mRNA levels and used to carry out quantitative PCR analysis to look at EGFR mRNA levels in these cell lines (Figure 16e). The BT20 cell line was used because it not only expresses EphA2, but it also has EGFR amplification. The basis for using this cell line was to ensure that there is ample EGFR for the EphA2 to interact with. Larsen *et al.* also reported that the EphA2 gene is a downstream transcriptional target of the EGF-activated EGFR pathway (100). However, when EGFR was transiently reduced in these same cell lines EphA2 receptor levels appeared unaffected (Figure 16c,d). Possible explanations for this will be in the discussion section.

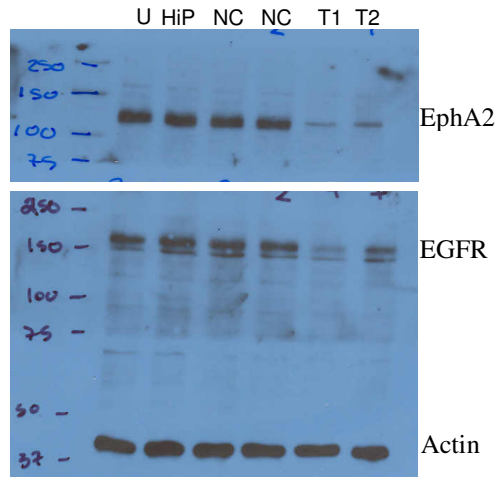
Figure 16. Transiently Reducing EphA2 Levels Leads to a Reduction in EGFR Expression

MDA-MB-231 Luc and BT20 cells were both treated with either EphA2 targeting siRNA (T1 and T2) or EGFR targeting siRNA (E1, E2, and E3) to transiently reduce levels of the corresponding protein. Membranes were then probed with EphA2 and EGFR antibodies to reveal the quantities of the receptors in the protein lysates. **(A)** MDA-MB-231 Luc cells treated with EphA2 siRNA (top blot) showed reduced levels of EphA2. Subsequent probing for EGFR on the same membrane revealed a reduction in EGFR protein levels also (middle blot). Actin was used as the control to ensure that the protein samples were loaded equally. **(B)** MDA-MB-231 Luc cells were treated with EGFR targeting siRNA that successfully reduced the EGFR protein levels (upper, right). When probed for EphA2, no differences in EphA2 protein levels were found after treatment with EGFR targeting siRNA (upper, left). Actin was used as the control to ensure that the protein samples were loaded equally. **(C)** EGFR targeting siRNA successfully reduced EGFR protein levels in BT20 cells. **(D)** EphA2 protein levels were unaffected by the transiently reduced levels of EGFR in BT20 cells. Actin was used as the control in both **C** and **D** to ensure equal protein loading. **(E)** Relative quantities of EGFR and EphA2 mRNA in a panel of breast cancer cell lines.

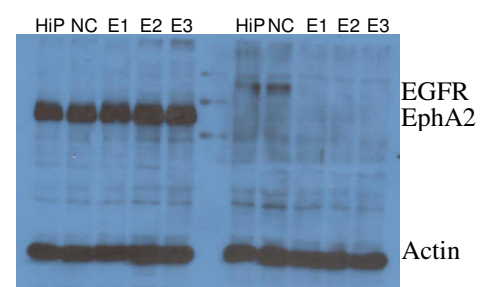
Figure 16

Transiently Reducing EphA2 Levels Leads to a Reduction in EGFR Expression

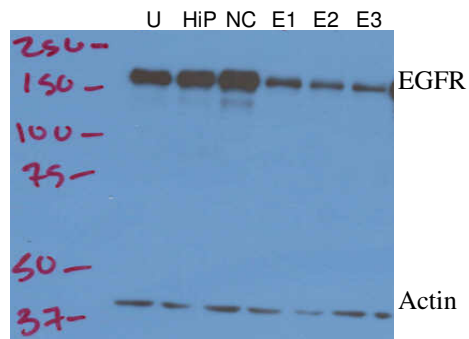
(A) MDA-MB-231 Luc: EphA2 siRNA



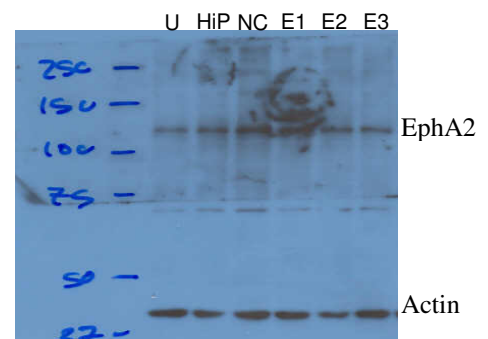
(B) MDA-MB-231 Luc: EGFR siRNA



(C) BT20: EGFR siRNA

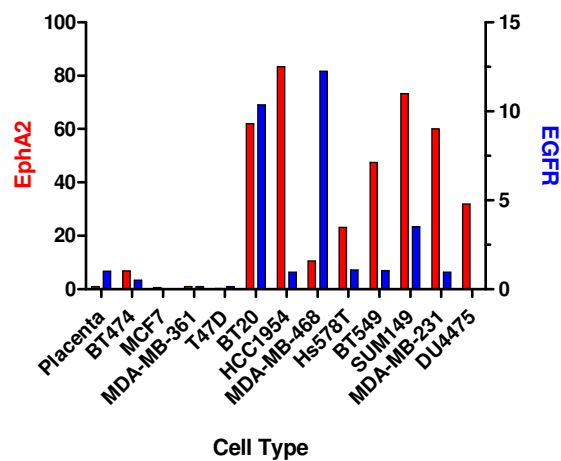


(D) BT20: EGFR siRNA



(E)

Relative Quantities of EGFR and EphA2 mRNA



EphA2 and EGFR Co-localize at the Plasma Membrane of MDA-MB-231 Luc Cells

Having seen that there is a relationship between EphA2 and EGFR protein levels when EphA2 is transiently reduced, it was hypothesized that EphA2 and EGFR may colocalize at the plasma membrane and could potentially interact within a complex. EphA2 and EGFR were found to be co-localized in the HN5 and A431 cell lines, evidence that further motivated investigation into this hypothesis (100). To visualize the location of EphA2 and EGFR in MDA-MB-231 Luc cells, immunocytochemical fluorescent staining was carried out (Figure 17a). Images revealed that EphA2 and EGFR do appear to colocalize in this cell line. To confirm and enhance the validity of this finding, cells were also stained so that they could be imaged using a confocal microscope which enables the cells to be reconstructed in three dimensions (Figure 17b). The technique of Z-stacking was employed which allows different planes at various depths within the cell to be visualized (Figure 17c). This represents a way to see if the cellular EGFR and EphA2 co-localization at the plasma membrane is consistent throughout the cell, and we found that it is. These fluorescent and confocal images revealing EphA2 and EGFR co-localization led us to propose that the two receptors could possibly be physically interacting.

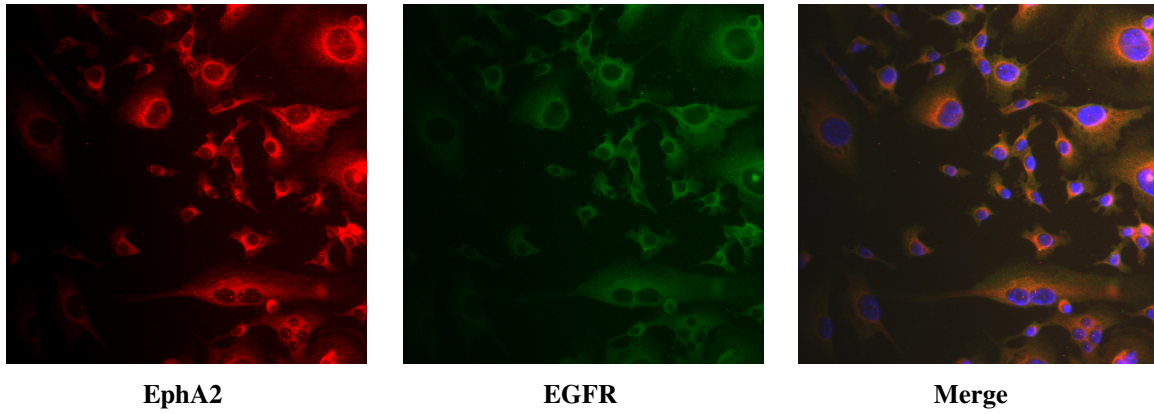
Figure 17. Demonstration of EphA2 and EGFR Co-localization

(A) MDA-MB-231 Luc and MCF7 (not shown) cells were stained for fluorescent imaging. MCF7 served as the negative control for the specificity of reagents seeing as they do not express EphA2 or EGFR. EphA2 and EGFR staining can be visualized on the plasma membrane, specifically in areas of membrane ruffling, and distributed throughout the cytoplasm. MCF7 cells have little to no endogenous expression of EphA2 and EGFR and, when stained for these two receptors, no positive staining occurred. (B) Confocal imaging of MDA-MB-231 Luc cells revealed co-localization of EphA2 (green, top right) and EGFR (red, top left). Nuclear staining is shown in blue (bottom left) and the merged image is shown in the bottom right square. (C) This image represents one of the Z-stack images showing EphA2 and EGFR co-localization. EGFR is shown in green (top left), EphA2 is shown in red (top right), nuclear staining is shown in blue (bottom left), and the merged image is shown in the bottom right square. Confocal and Z-stack images were also compared to stained MCF7 cells which did not show positive expression of EphA2 and EGFR.

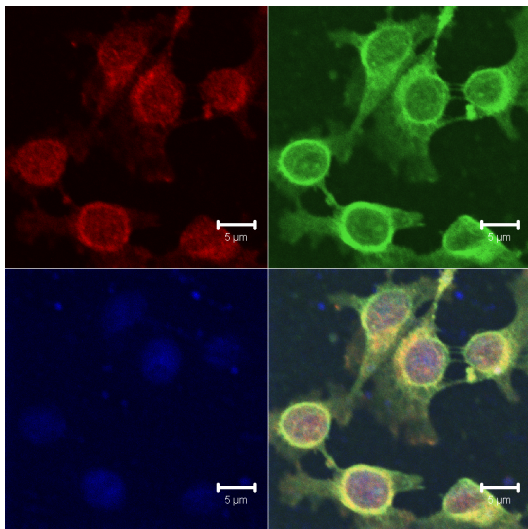
Figure 17

Demonstration of EphA2 and EGFR Co-localization

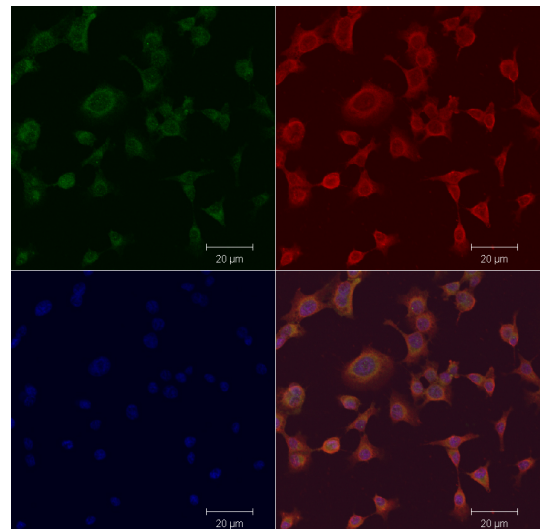
(A) Fluorescent Imaging



(B) Confocal Imaging



(C) Confocal Imaging: Z-Stacking



Immunoprecipitation of EphA2 from MDA-MB-231 Luc Cell Protein Lysates Did Not Pull Down of EGFR After Stimulation with EGF

Western blot analyses had previously revealed that EGFR protein levels are reduced when cells are treated with EphA2-targeting siRNA. This led to the proposal that EGFR and EphA2 may co-localize at the plasma membrane and interact within a complex.

Disruption of the physical interaction between EGFR and EphA2 due to transient reduction of EphA2 levels with siRNA might destabilize EGFR at the membrane leading to subsequent internalization and degradation of the receptor. To investigate whether or not EphA2 and EGFR complex, co-immunoprecipitation assays were completed. Equal amounts of protein from MDA-MB-231 Luc cells that were either stimulated with 50ng/ml EGF (+) or left un-stimulated (-) were immunoprecipitated with 5µg of mouse anti-Eck antibody. Agarose beads were then used to precipitate any immunocomplexes. Western blot analysis revealed that immunoprecipitation of EphA2 by an anti-EphA2 antibody does not lead to the pull down of EGFR in these breast cancer cells (Figure 18a,b).

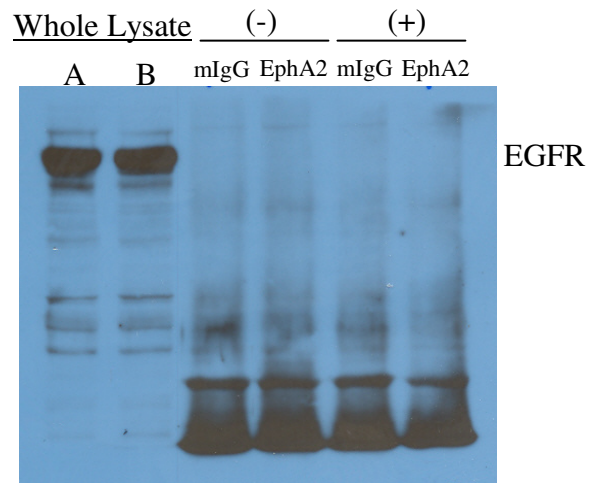
Figure 18. Immunoprecipitation of EphA2 Does Not Lead to EGFR Pull Down

(A) MDA-MB-231 Luc cell pre-cleared protein lysates were immunoprecipitated with either a negative control mouse IgG antibody or anti-EphA2 antibody. Subsequent western blot analysis revealed that EGFR had not been pulled down. 20µg of the original, non-immunoprecipitated protein lysate was set aside as a control to ensure that EGFR was present. (B) The membrane was stripped and reblotted for EphA2 to ensure that it had been immunoprecipitated by the anti-EphA2 antibody. EphA2 was both present in the original whole lysate and was also successfully precipitated out. Results are representative of multiple experiments.

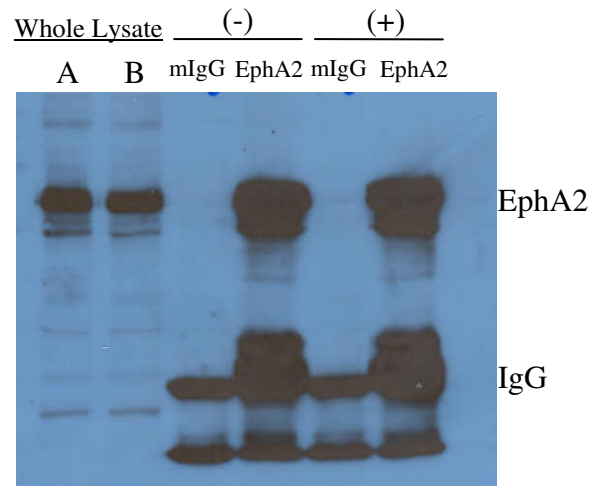
Figure 18

Immunoprecipitation of EphA2 Does Not Lead to EGFR Pull Down

(A)



(B)



Comparison of Vascular Endothelial Growth Factor Release in Cells Treated with Non-Targeting siRNA and EphA2-Targeting siRNA

Angiogenesis is one of the key processes required for successful tumor establishment and growth (67). Recruitment of preexisting vessels and development of new vessels within the tumor hinges upon proper expression and function of pro-angiogenic growth factors and their receptors (67, 112, 113). Eph receptors and their ligands have been implicated to be involved in the development of embryonic vasculature (114). Recently, studies have revealed that EphA2 is important in the tumor microenvironment in order for angiogenesis to occur for progression to metastasis (113). Furthermore, endothelial cells lacking EphA2 do not participate in vascular assembly, nor do they participate in vascular migration (115). Although EphA2 appears to be associated with the process of vessel formation and growth during tumorigenesis, the specific mechanism behind this association remains vague. Release of the vascular endothelial growth factor (VEGF) was measured in breast cancer cell lines with reduced EphA2 to establish whether the receptor protein reduction correlated with a decrease in VEGF release. Preliminary data suggested that transiently reducing the EphA2 levels in MDA-MB-231 cells produced a significant decrease in VEGF release into the supernatant (Figure 19a). However, repetition of this experiment did not reproducibly find a strong correlation between EphA2 receptor depletion and decreased VEGF release in the MDA-MB-231 cell line, HCC1954 cell line, nor the BT549 cell line (Figure 19b,c).

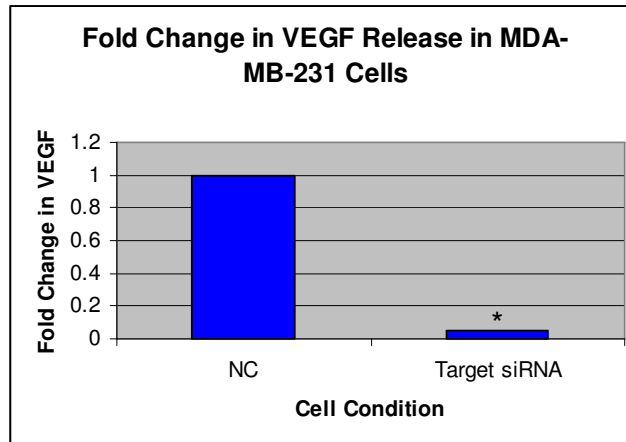
Figure 19. VEGF Release in Metastatic Breast Cancer Cell Lines with Attenuated Levels of EphA2 Receptor Protein Levels

(A) ELISA analysis of supernatant collected from MDA-MB-231 cells after treatment with EphA2-targeting siRNA revealed lower levels of released VEGF compared to cells treated with the non-targeting siRNA or with the transfection reagent alone (preliminary result). This data was found to be statistically significant using the GraphPad Prism 5 software. (B) ELISA analysis of supernatant collected from HCC1954 cells after treatment with EphA2-targeting siRNA revealed mildly lower levels of released VEGF compared to cells treated with the non-targeting siRNA or with the transfection reagent alone. These data were not statistically significant. (C) ELISA analysis of supernatant collected from BT549 cells after treatment with EphA2-targeting siRNA revealed no reduction in released VEGF compared to cells treated with the non-targeting siRNA or with the transfection reagent alone.

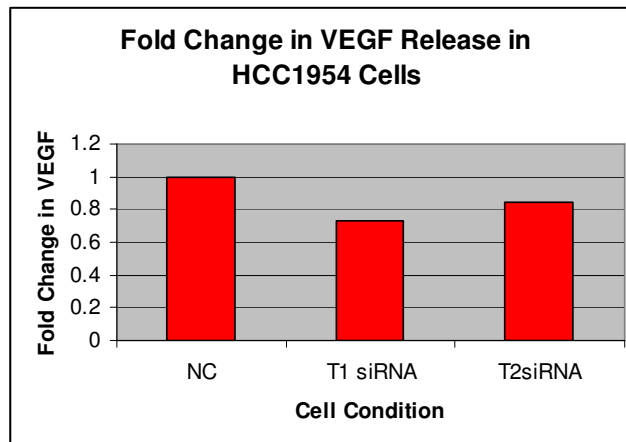
Figure 19

VEGF Release in Metastatic Breast Cancer Cell Lines with Attenuated Levels of EphA2 Receptor Protein Levels

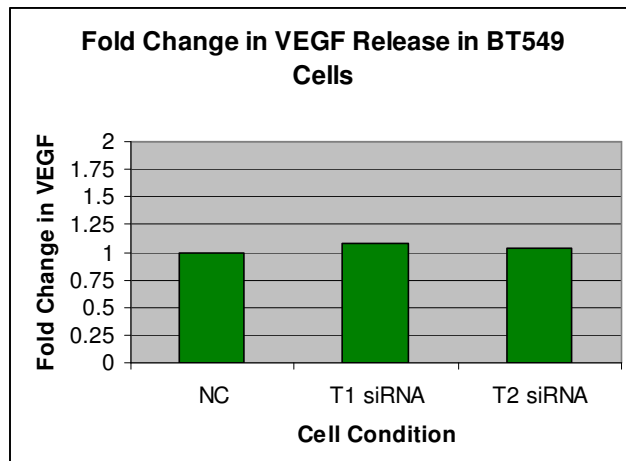
(A)



(B)



(C)



Results: *In vivo* Studies

Having accumulated some data supporting the hypothesis that transiently depleted EphA2 levels will negatively affect *in vitro* assays that indicate tumor cell metastatic capability, we wanted to see if reducing EphA2 levels with siRNA would have any effect on established mammary fat pad tumor growth and persistence in female athymic nude mice.

Since its discovery, the use of the RNA interference (RNAi) pathway as a method of silencing the expression of specific genes has continually helped to advance our understanding of basic biology and disease states (116). Introducing siRNA into a cell results in its uptake and incorporation into the RISC (RNA-induced silencing complex) protein complex which recognizes complementary mRNA in the cytoplasm and destroys them (116). While RNAi is a natural part of eukaryotic processes, artificially synthesizing and efficiently delivering siRNA to its specific target in a live animal model has proven to be a challenge (116).

However, there are two methods of siRNA delivery that have been reported to be successful and that we employed to deliver non-targeting or EphA2-targeting siRNA to the mammary fat pad tumors in our experiments. The first are DOPC liposomes. DOPC is a natural lipid used to synthesize liposomes. siRNA can be packaged within DOPC liposomes and injected for intratumoral delivery (95, 107). We also used chitosan nanoparticles as a route of siRNA delivery because, among other things, they are unlikely to elicit an immune response and they are nontoxic (117). Furthermore, nanoparticles have been shown to be more efficient than DOPC liposomes because they have a positive

charge that allows for increased transport across the cell membrane (118). Additionally, Han *et al.* showed that certain ligands can be attached to the nanoparticles in order to specifically target markers found on the surface of tumor cells (118). In their case, they used the RGD ligand (Arg-Gly-Asp) which targeted the $\alpha v \beta 3$ integrin expressed on ovarian cancer cells (118). This technique could possibly be applied to specifically target EphA2 on the surface of breast cancer cells. In addition to delivering siRNA (either EphA2 targeting or non-targeting), we also designated some groups to be treated with chemotherapy as well. In total we had ten groups: PBS Control, PBS + Paclitaxel, DOPC Control siRNA, DOPC Control siRNA + Paclitaxel, DOPC EphA2 siRNA, DOPC EphA2 siRNA + Paclitaxel, Nanoparticle Control siRNA, Nanoparticle Control siRNA + Paclitaxel, Nanoparticle EphA2 siRNA, Nanoparticle EphA2 siRNA + Paclitaxel. Those mice that were also receiving chemotherapy were injected i.p. once weekly with 24mg/kg of Paclitaxel. This dose was established to be effective, yet non-toxic, in preliminary studies carried out by previous laboratory members.

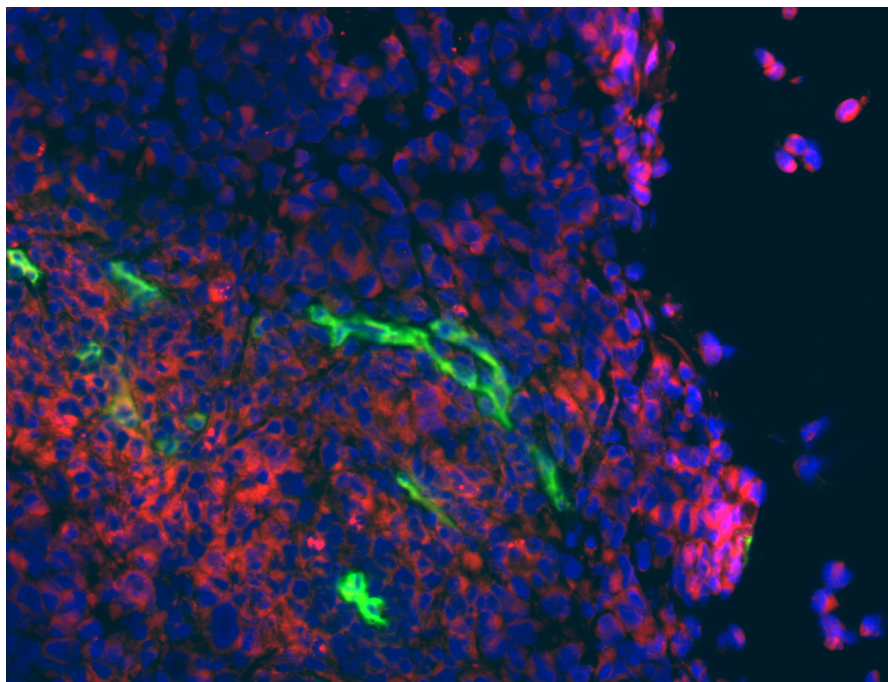
Previous research has shown that fluorescently labeled DOPC liposomes injected i.v. into mice were detected in mammary fat pad tumors of the MDA-MB-231 breast cancer cell line (Price *et al.*, unpublished data). This observation and the findings with a melanoma xenograft model supported the feasibility of systemic administration to deliver siRNA to solid tumors (119) (Figure 20).

**Figure 20. Successful Delivery of Fluorescently Labeled DOPC Liposomes to MFP
Tumors**

A cross section of a MDA-MB-231 Luc established mammary fat pad tumor collected 6 hours post i.v. injection of fluorescently labeled DOPC liposomes. CD31 staining is represented by the green color, tumor cell nuclei staining is represented by the blue color, and the fluorescently tagged DOPC liposomes are represented by the red color.

Figure 20

Successful Delivery of Fluorescently Labeled DOPC Liposomes to MFP Tumors



(Price *et al.*, unpublished data)

Delivery of EphA2-Targeting siRNA by DOPC Liposomes or Chitosan

Nanoparticles had No Significant Impact on Tumor Growth to the Corresponding Control

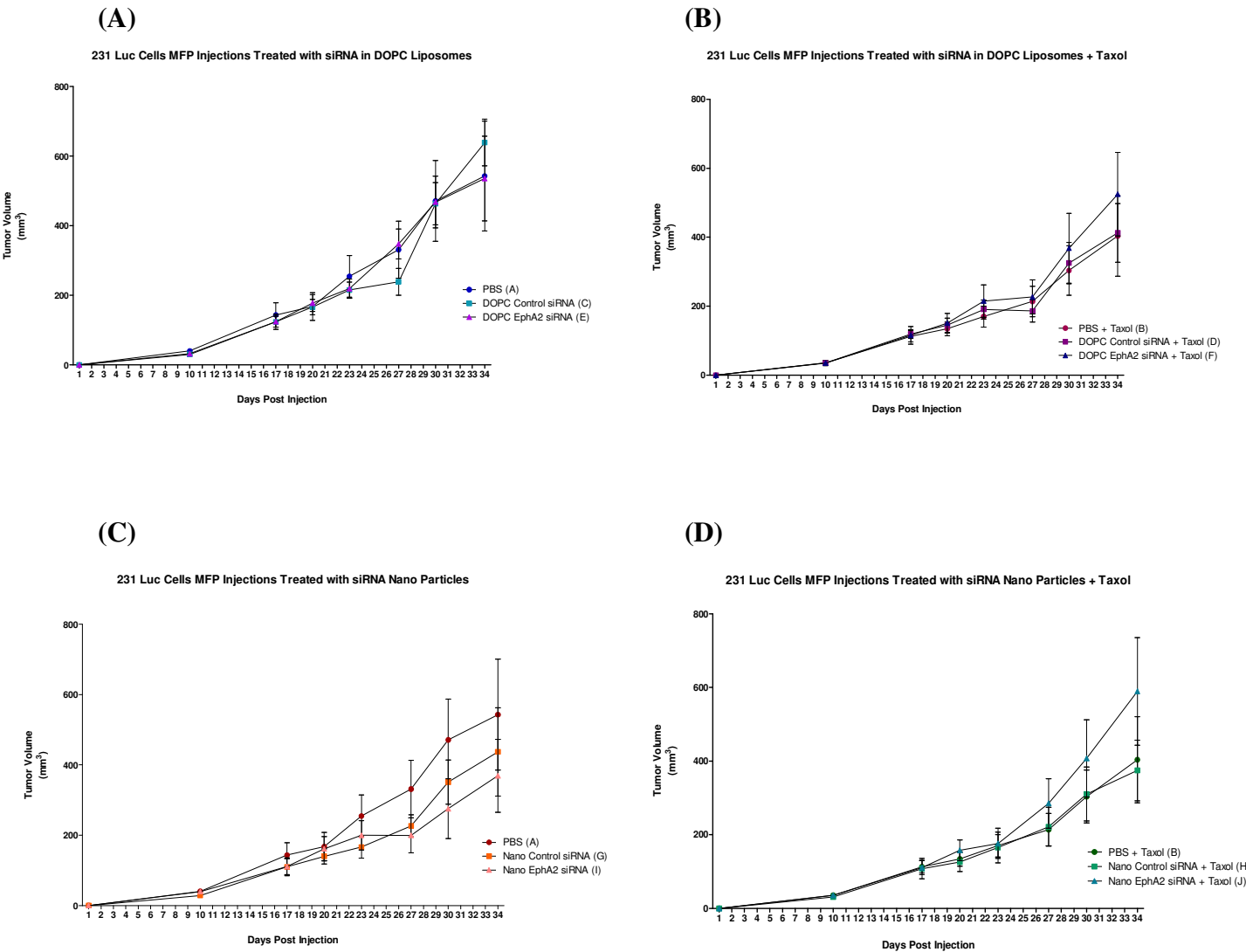
MDA-MB-231 Luc cells were injected into the mammary fat pad of female nude mice and allowed to grow. Once the tumors became palpable, they were measured twice a week and the volumes were calculated. Tumor growth for each group was monitored over time and recorded as volume vs. time (Figure 21a,b,c,d). Additionally, the dates and numbers of mice sacrificed were noted and analyzed by means of a survival curve (data not shown). Although there was no significant difference in tumor sizes, the mice treated with EphA2-targeting siRNA nanoparticles without paclitaxel treatment did show a modest decrease in tumor growth and had a longer mean survival time than the comparative control (Figure 21c). The dose of paclitaxel used in these experiments had previously been shown to control tumor growth and be non-toxic, but it was not effective in this experiment. Although monitoring tumor growth and animal survival are useful ways to analyze the effect of EphA2 reduction in MFP tumors *in vivo*, a more direct readout of the effect of EphA2-targeting siRNA was measured by analyzing EphA2 protein content in the tumor tissues.

Figure 21. Tumor Growth in siRNA Treated Animals

Mammary fat pad tumors were monitored and their lengths and widths were recorded as they grew. The tumor volumes were calculated and plotted on a graph versus time. Day 0 represents the day the cells were first injected into the mammary fat pad of the mice and all subsequent days represent the number of days post-injection. Tumor volumes of a particular experimental group receiving EphA2-targeting siRNA were compared to the corresponding control group receiving the non-targeting siRNA and the PBS control group. Tumor growth can be seen in the DOPC liposome group (**A**), the DOPC liposome + Paclitaxel group (**B**), the nanoparticle group (**C**), and the nanoparticle + Paclitaxel group (**D**). According to these graphs, there is no significant difference in tumor growth compared to the respective controls in any of the groups, however, the mice treated with EphA2-targeting nanoparticles without Paclitaxel revealed decreased tumor growth compared to the controls. The p-value for this group was 0.5. Arrows indicate when treatment was initiated, which was 21 days after MFP injection.

Figure 21

Tumor Growth in siRNA Treated Animals



Analysis of Tumor Protein Lysate Revealed EphA2 Protein Levels Parallel the Trend Seen in Tumor Growth between the Various Experimental Groups

At the time of animal sacrifice, tumor tissue was collected for protein analysis. Snap frozen tumor tissue was lysed, purified, and sampled for EphA2 protein quantities.

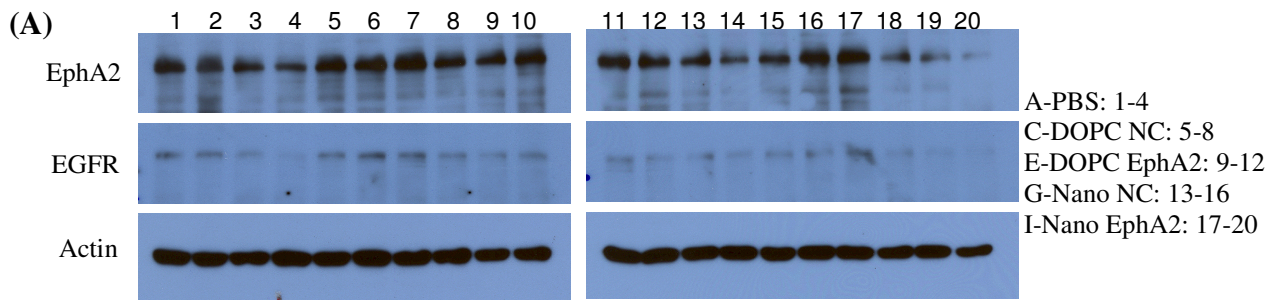
Western blot analysis revealed that all tumors without paclitaxel treatment had uniformly strong EphA2 expression, the exception being those samples collected from the group treated with EphA2-targeting siRNA chitosan nanoparticles (Figure 22a). This group had the lowest amounts of EphA2 expression and also had decreased tumor growth over time. Further densitometry analysis of EphA2 protein levels confirmed a slight reduction in the tumor tissue taken from those tumors where mice had been treated with EphA2-targeting chitosan nanoparticles in comparison their respective control (Figure 22b). Actin levels were blotted for as a control to ensure equal protein loading. Due to the lack of significant differences amongst those groups that also received concomitant chemotherapy, we only analyzed tumor EphA2 protein levels in the groups treated with the nanoparticles and paclitaxel (Figure 22c). In these particular groups, no EphA2 protein reduction was seen, and in fact, EphA2 levels were higher in these tissue lysates when compared to any of the DOPC liposome and nanoparticle treated groups that didn't receive paclitaxel. All protein lysates samples were also analyzed for levels of EGFR protein, but no correlation or trend was found from this protein analysis.

Figure 22. EphA2 and EGFR Protein Levels in Animal Tumor Tissue Treated with PBS, DOPC Liposomes, and Chitosan Nanoparticles

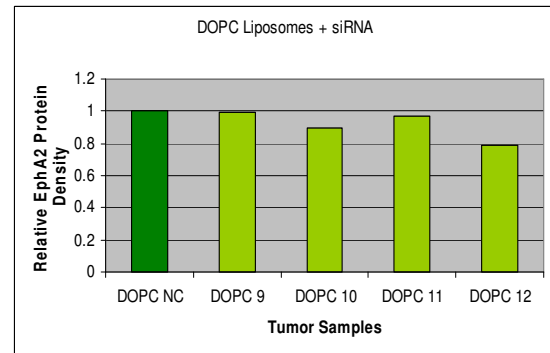
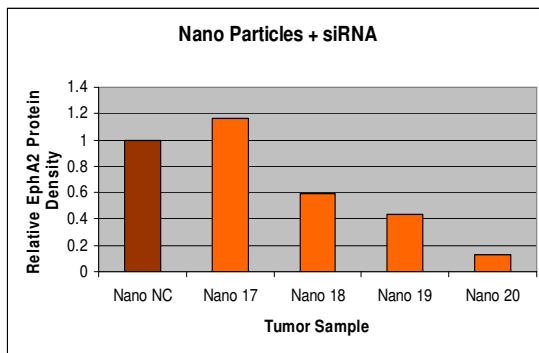
(A) Western blot analysis of protein isolated from the mammary fat pad tumors collected from the mice treated with PBS, DOPC liposomes containing either EphA2-targeting siRNA or non-targeting siRNA, or chitosan nanoparticles containing either EphA2-targeting siRNA or non-targeting siRNA revealed no obvious attenuation in EphA2 protein levels except in the group of tumors where mice were treated with EphA2-targeting nanoparticles. Immunoblotting for EGFR did not reveal a trend or correlation with EphA2 protein levels. Immunoblotting for actin served as the control to ensure that equal amounts of protein were loaded. (B) Densitometry of the blot confirmed that EphA2 protein levels were lower in the tumors where the mice were treated with EphA2-targeting nanoparticles in comparison to the corresponding control (left). The EphA2 protein levels in those mice treated with EphA2-targeting DOPC liposomes were not attenuated and were equivalent to the levels seen in the corresponding control (right). (C) Protein analysis of tumors that were treated with both EphA2-targeting nanoparticles and Paclitaxel showed enhanced EphA2 expression compared to the corresponding control (left). Actin was blotted for to ensure equal protein loading amongst samples. Densitometry analysis confirmed this enhanced expression (right).

Figure 22

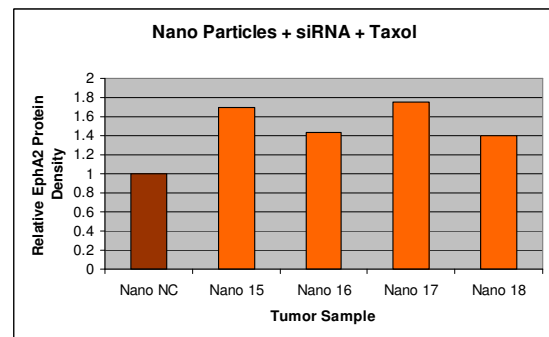
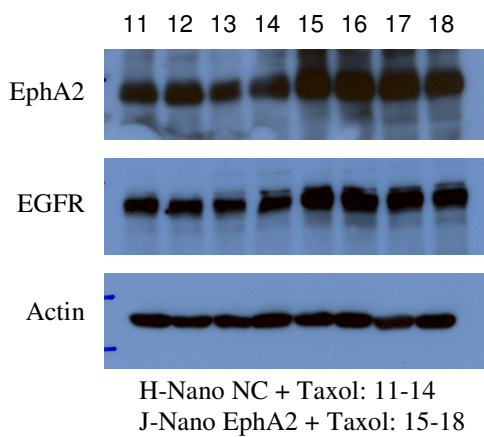
**EphA2 and EGFR Protein Levels in Animal Tumor Tissue Treated with PBS,
DOPC Liposomes, and Chitosan Nanoparticles**



(B)



(C)



Discussion: Utilization of Targeted siRNA to Study the Effect of Transient EphA2 Reduction *In vitro* and *In vivo*

The goal of Specific Aim II was to utilize EphA2-targeting siRNA to study the effect of reduced receptor levels on properties indicating aggressive and metastatic behavior in metastatic breast cancer cell lines *in vitro* and in mammary fat pad tumors *in vivo*. EphA2 overexpression was found in the basal subtype of breast cancers at both the protein and mRNA levels. Breast cancer cell lines of the basal subtype are clinically more aggressive and are most infamously known for exhibiting the “triple negative” phenotype (7, 33). This correlates with data obtained from studies done in other cancer models where EphA2 overexpression is found in the more aggressive and metastatic cancer cell lines (64, 66, 69).

The ability of tumor cells to migrate positively correlates with metastatic potential because migration is crucial for tumor cell invasion, a key step in the ability of tumor cells to escape from the confines of the primary tumor and enter into circulation (89, 120). EGFR activation by its ligand, EGF, is one example of a receptor-ligand association that is a pertinent part of the migratory capability of tumor cells (100, 120). Larsen *et al.* have shown that EphA2 is a transcriptional target of the activated EGF-EGFR pathway and that the two receptors physically interact to induce cell motility (100). Treatment of BT549 and MDA-MB-231 Luc cells with EphA2-targeting siRNA resulted in attenuated migration towards the EGF chemoattractant (Figure 15). It was therefore hypothesized that EphA2 and EGFR physically interact in order to stimulate tumor cell migration in breast cancer cells. This hypothesis seemed logical given that a reduction in EphA2

protein levels might destabilize EGFR at the plasma membrane, leading to increased EGFR internalization and degradation (Figure 16). With less EGFR present on the cell surface, breast cancer cells would be unable to migrate as efficiently in the presence of EGF compared to those cells where EphA2 had not been transiently knocked down. However, co-immunoprecipitation studies using protein lysates from either unstimulated or EGF-stimulated MDA-MB-231 Luc cells failed to show that precipitation of EphA2 by an anti-Eck antibody would pull down EGFR (Figure 18). There are several possible reasons why this data did not confirm what Larsen *et al.* had found. Firstly, the A431 cell line used by Larsen's group has significantly higher physiological levels of EGFR than the MDA-MB-231 Luc cell line due to EGFR amplification. A quantification of EGFR receptor levels found on the cell surface of both of these cell types indicates that the EGFR levels on the A431 cells is at least 8 fold higher than the EGFR levels found on the surface of MDA-MB-231 Luc cells (121, 122). This receptor amplification may affect the co-immunoprecipitation of EphA2 and EGFR. It is possible that although MDA-MB-231 Luc cells do express EGFR, the endogenous levels are not high enough to exhibit the EGFR-EphA2 complex formation by co-immunoprecipitation analysis. Furthermore, the MDA-MB-231 Luc cells were stimulated with EGF for 15 minutes before harvesting the cells and collecting the protein lysate. The A431 and HN5 cell lines in the work of Larsen *et al.* were stimulated with EGF for 24 hours before carrying out the co-immunoprecipitation assay. It's possible that the MDA-MB-231 Luc cells should be stimulated with EGF for a longer period of time in order to successfully demonstrate EGFR pull down after precipitation of EphA2. This would be something to look into for future experiments.

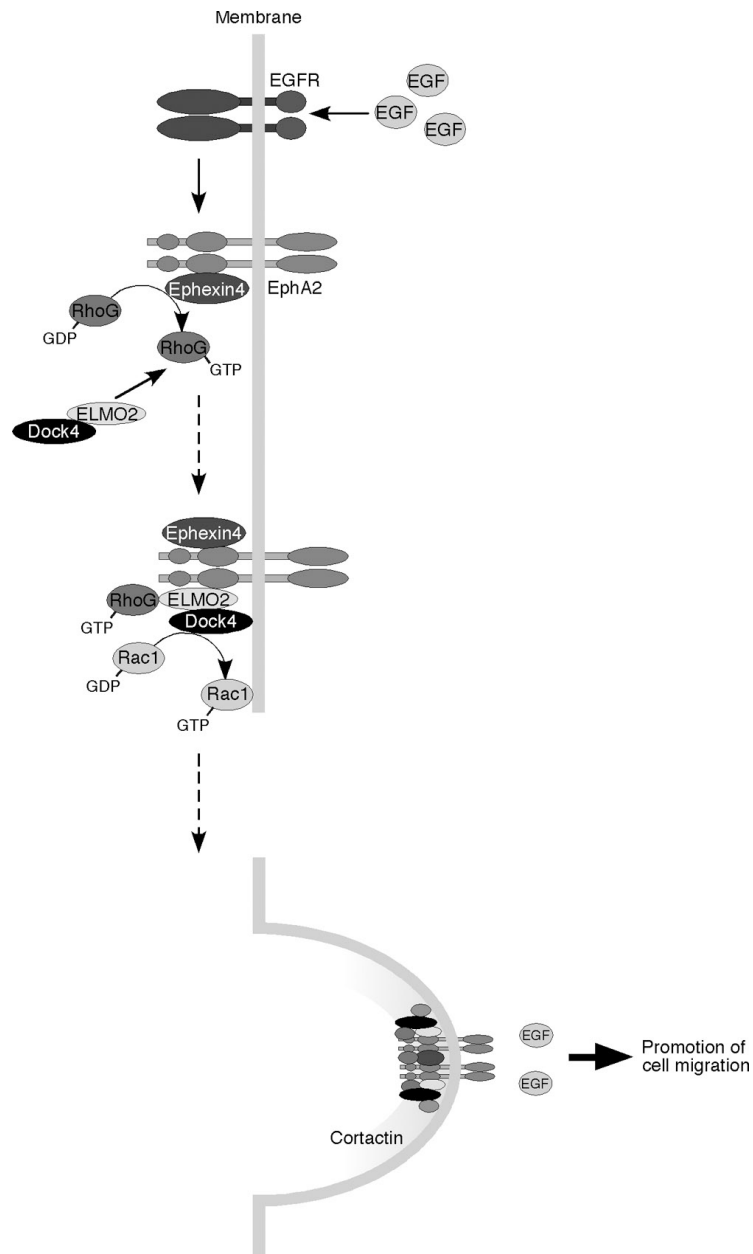
While the possible interaction of EGFR and EphA2 within a complex should certainly continue to be a focus of investigation, a couple of recent studies have indicated that EGFR and EphA2 may both be vital players in the migratory mechanics of breast cancer cells without necessarily physically interacting (102, 103). Miao *et al.* used the glioblastoma cell line, U373, to show that EGF-stimulated EGFR results in the activation of Akt. When EphA2 is not being stimulated by its own ligand, ephrinA1, it serves as a substrate for this EGFR-activated Akt leading to serine phosphorylation of EphA2 (102). Additionally, Miao *et al.* were able to show that inhibiting either the activation of Akt by EGFR or the serine phosphorylation of EphA2 by activated Akt resulted in decreased cell migration, however, the downstream mechanism leading to migration was not elucidated (102). More recently, Hiramoto-Yamaki *et al.* have produced data using breast cancer cells that suggests that in response to EGF stimulation, Ephexin4 binds EphA2 leading to RhoG activation and recruitment of ELMO2 and Dock4 to the Ephexin4-EphA2 protein complex (103) (Figure 16). This activated complex activates Rac1 and leads to the promotion of cell migration (103). They have yet to unveil how EphA2 is being activated that is leading to Ephexin4 recruitment, but they suggest it could be the same Akt mechanism that was shown to promote cell migration through EphA2 in the glioblastoma cells (102, 103). Linking these two mechanisms has recently been the subject of my investigations in metastatic breast cancer cell lines as a way to explain how EphA2 is involved in the promotion of tumor cell migration. According to this hypothesis, siRNA-mediated EphA2 reduction would certainly attenuate the migratory ability of breast cancer cells by inhibiting the formation of the Ephexin4-EphA2-Elmo2-Dock4 protein complex that ultimately activates Rac1 (Figure 16).

Figure 23. Proposed Mechanism of EphA2's Role in Rac1 Activation and Cell Migration

The proposed mechanism of EphA2 involvement in cancer cell migration begins with the stimulation of EGFR by its ligand EGF. Once activated, EphA2 becomes activated by an unknown mechanism. Ephexin4 is recruited to EphA2 the activated complex stimulates the RhoG exchange of GDP for GTP so that it is now in the activated state. Activated RhoG recruits Elmo2 and Dock4 to the protein complex. This protein complex stimulates the Rac1 exchange of GDP for GTP, thus activating it. The entire activated complex localizes at points of membrane ruffling to promote cell migration.

Figure 23

Proposed Mechanism of EphA2's Role in Rac1 Activation and Cell Migration



Whether EGFR and EphA2 are interacting within a complex to promote cell migration, or whether Akt is acting as the intermediate between the two receptors, there were still other dynamics of the EphA2-EGFR relationship to be explored. EphA2 has been shown to be a transcriptional target of EGF-activated EGFR (100). To see if the same relationship existed in breast cancer cell lines and to see if the opposite might be true (EGFR is a transcriptional target of EphA2), MDA-MB-231 Luc and BT 20 cells were treated with either EGFR or EphA2-targeting siRNA. In both cell lines, transient reduction of EphA2 led to a decrease in EGFR, but transient reduction of EGFR did not lead to a reduction in EphA2 (Figure 16). It's possible that, even though we cannot detect a direct association by the co-immunoprecipitation assay, the reduction in EphA2 leads to increased EGFR internalization and degradation. It's also possible that the activated EphA2 pathway in cancer cells drives EGFR at the transcriptional level, and a reduction of EphA2 attenuates that upregulation. However, this cannot be conclusively determined using only siRNA methods; rather, investigating whether EphA2 signaling has an impact on EGFR promoter activity would be more indicative whether EphA2 can regulate EGFR expression.

At first it was puzzling to see that EGFR reduction did not lead to decreased EphA2 levels, as suggested by work from other groups, but it was then realized that MDA-MB-231 Luc and BT20 cells are unique. First, MDA-MB-231 Luc cells have a constitutively activated Ras that activates EphA2 transcription (77). If the EGFR pathway does lead to EphA2 upregulation, siRNA-mediated reduction of EGFR wouldn't necessarily affect EphA2 in this cell line because the constitutively activated Ras would maintain the overexpression of EphA2 independent of EGFR (77). Additionally, BT20

cells have amplified EGFR. Even with the EGFR-targeting siRNA, the levels of endogenous EGFR may be so high that it is not being reduced enough and transcriptional upregulation of EphA2 is still occurring (123). In order to clarify what the true relationship is between EGFR and EphA2 in breast cancer cells after transient reduction of either receptor, it would be prudent to try other metastatic breast cancer cell lines that do not possess these unique alterations. Moreover, it is also important to note that inhibition of migration after transient reduction of EphA2 using siRNA was not only seen with EGF used as a chemoattractant. Cells with transiently reduced EphA2 also showed reduced migration towards CXCL12, indicating that while there may be a relationship between EGFR and EphA2 that is modulating cancer cell migration, EphA2 also works through other pathways to affect migration (Price *et al.*, unpublished).

Lin *et al.* has indicated that EphA2 plays a large role in the angiogenic process in ovarian cancer and it was thus hypothesized that EphA2 may play a similar role in metastatic breast cancers (66). Measuring the concentration of VEGF release in the supernatant of cultured cells is one indication of cellular participation in angiogenesis because this growth factor has been found to be necessary for the recruitment and persistence of intra-tumoral vessels (124). However, although preliminary studies revealed that decreased EphA2 expression in MDA-MB-231 cells causes a significant diminution in the amount of VEGF released, this result could not be repeated to achieve the same level of significant reduction in VEGF release in either the MDA-MB-231 cells or two other metastatic breast cancer cell lines (Figure 19). While this data is discouraging, it's possible that quantifying cellular release of VEGF is not the most informative way to elucidate the effect of reduced EphA2 expression in the angiogenic

process in breast cancer cells. Lin *et al.* focused on examining matrix metalloproteinase (MMP) expression in EphA2 overexpressing ovarian carcinomas where they found a trend between high EphA2 expression and high MMP expression (124). The expression of MMPs in metastatic breast cancer cells in the presence or absence of EphA2 was not examined in my study and may be worthwhile to investigate in the future due to their established role in the process of angiogenesis (105).

The conclusion that can be made from the *in vivo* studies whereby EphA2-targeting siRNA was delivered either in DOPC liposomes or chitosan nanoparticles with or without paclitaxel is that EphA2 siRNA nanoparticle delivery seemed to be superior to delivery by DOPC liposomes, but only when delivered alone and not in conjunction with chemotherapy (Figures 21). The superiority of nanoparticles to DOPC liposomes in successfully delivering siRNA to our mammary fat pad tumors confirms what others have found in other cancer models (96, 118). The experiment conclusions were made primarily based on the analysis of the tumor tissue protein (Figure 22). EphA2 protein was most reduced in the mammary fat pad tumors that had been treated with EphA2-targeting siRNA in chitosan nanoparticles and this is also the group that showed a trend towards decreased tumor growth. EphA2 did not seem to be reduced in the tumors of the other treatment groups and this correlated with a lack of difference in tumor growth between treatment groups and control groups. The lack of reduction in EphA2 protein levels seen in the majority of the treatment groups could be attributed to a couple of different explanations. Firstly, it's not known if the DOPC liposomes containing the EphA2-targeting siRNA succeeded in reaching the mammary fat pad tumors. It was assumed that they did based on preliminary data revealing fluorescently labeled siRNA in DOPC

liposomes distributed throughout sections of the tumor tissue (Figure 20). This cannot be confirmed for this particular experiment since the siRNA was not tagged this time.

Assuming the EphA2-targeting siRNA did reach the tumors, this suggests that more siRNA needs to be delivered more frequently in order to have a significant impact on negative impact on intratumoral EphA2 protein levels and mammary fat pad tumor growth. Furthermore, it is not known whether the delivered siRNA had off-target effects.

Overall, the reduction of EphA2 after utilization of targeting siRNA reveals a negative association between attenuated EphA2 protein levels and indicators of aggressive and metastatic tumor cell behavior. Although this trend is quite clear, there is much investigation that still needs to be done to elucidate the specific mechanisms which are associated with EphA2 reduction and decreased metastatic potential.

- Chapter 5 -
Summary & Conclusions

Summary

The development of any given type of cancer requires genetic and/or epigenetic alterations that change the normal function of cells, giving them the ability to evade homeostatic mechanisms that normally regulate cell activities (125). This may lead to atypical cellular proliferation and subsequent development of benign or malignant tumor formation (85). Tumor formation occurs in nearly every location within the human body and proof of this is seen in the high number of newly diagnosed cancer cases each year, both nationally and internationally (1). This has led to a worldwide investigation into the process of cancer development, establishment, and metastasis (1, 85). Metastasis itself is a complex process often involving genetic changes in addition to the original cancer-initiating mutations (85). These acquired cellular alterations allow tumor cells to invade into the surrounding tissue and migrate to the closest vessels where they enter the circulation and may establish secondary tumors elsewhere (metastases) (85). The importance of tumor cells having the capability to metastasize is that these metastases are the primary cause of cancer-related deaths in solid tumors due to the fact that they often establish themselves in critical locations such as the lungs, brain, and bones (85).

EphA2 has been identified in many cancers to be a receptor tyrosine kinase that is overexpressed in the more aggressive forms of the diseases (53, 61). This is true for melanomas, ovarian cancer, lung cancer, and prostate cancer (63, 65, 126). It has been found to increase the metastatic ability by participating in angiogenesis, migration, invasion, and growth in these tumor models (63, 65, 66, 126). Importantly, EphA2 is shown to be expressed not only in breast cancer cell lines, but also in clinical tumor

samples (53). The implication is that studying the biology of EphA2 participation in the metastatic breast cancer model may prove to be important clinically as well. Furthermore, EphA2 is consistently found to be expressed in the aggressive, triple-negative breast cancers which are the most aggressive, the most likely to metastasize, and which have limited treatment options due to their lack of molecular targets such as ER and HER2 (7, 33, 77). Despite the inefficient process of metastasis and the number of hindrances tumor cells must overcome in order to successfully metastasize, metastasis is responsible for over 90% of human cancer deaths (127). Since breast cancer deaths are included in this percentage and because EphA2 is highly overexpressed in metastatic breast cancers, it is a prospective target for investigation (127). While studies have been done to establish which breast tumors and cell lines express EphA2, little has been done to indicate the role that EphA2 plays in these aggressive and metastatic breast cancers. Therefore, it was hypothesized that reduced EphA2 receptor levels in metastatic breast cancer cell lines and in established mammary fat pad tumors would negatively affect the metastatic tumor cell characteristics *in vitro* and would decrease tumorigenicity and tumor persistence *in vivo*, respectively.

Cancer cells are intriguing in that they must acquire several characteristics in order to successfully establish a tumor (67). They must both be self-sufficient in their production of growth signals, while being insensitive to anti-growth signals (67). They must also be able to evade intracellular cell death signals in order to proliferate without limits (67). Finally, cancer cells must have a self-sustained angiogenic capability and should be characterized by their ability to invade in order to metastasize (67). One way to study how EphA2 participates in these processes is to investigate what happens to

metastatic breast cancer cells when EphA2 expression has been diminished. EphA2-targeting siRNA and EphA2 shRNA sequences were used to achieve a transient and stable knockdown of EphA2 in the MDA-MB-231 Luc cell line, the BT549 cell line, and occasionally in the HCC1954 cell line (Figures 6 & 13). These three cell lines represent metastatic breast cancer cell lines that overexpress EphA2 at both the protein and mRNA levels.

Migration assays carried out in the presence or absence of chemoattractants when EphA2 expression was either maintained or depleted by siRNA or shRNA revealed that metastatic breast cancer cell migration was attenuated in the presence of the chemoattractants in cells where EphA2 receptor levels had been reduced (Figures 7 & 15). The ability of breast cancer cells to migrate is crucial to their ability to metastasize because they must be able to move to nearby vessels in order to intravasate and enter the circulation (67). These findings indicate that EphA2 is a participant in the acquired ability of metastatic breast cancer cells to migrate. Further investigation into the mechanism behind this participation has revealed that EGFR and EphA2 co-localize and seem to have a non-physical relationship in the promotion of tumor cell migration. Additionally, recent data from another group has indicated that the migration mechanism in breast cancer cells and other cancer cell types begins with EGFR stimulation by ligand, which subsequently results in the serine phosphorylation of EphA2 by Akt (102). This has yet to be confirmed by my studies. However, another recent study shows that EphA2 serine phosphorylation results in the downstream activation of Rac1 which promotes cell migration (103). Recent results that I have obtained reveal decreased Rac1 activation in the EphA2 silenced cell lines in the presence of EGF compared to the control cells, which

showed increased Rac1 activity after EGF stimulation. Although this observation does not directly connect the two mechanisms found by Miao *et al.* and Hiramoto-Yamaki *et al.*, it does indirectly link EGF stimulation of EGFR to increased Rac1 activation through EphA2. In order to better confirm this hypothesized pathway, it would be helpful to look at EphA2 serine phosphorylation in the metastatic breast cancer cell lines in the presence or absence of an Akt inhibitor and concurrently, in the presence or absence of EGF stimulation. Furthermore, it would be beneficial to confirm the decreased Rac1 activation in cells with reduced EphA2 expression by also looking at the effect of EGF stimulation in the presence or absence of Akt inhibitors on upstream participants of this proposed pathway, such as Ephexin4, in these cells. Unfortunately, antibodies against serine phosphorylation in EphA2 and against Ephexin4 are not commercially available and these studies could not be completed.

The siRNA-mediated and shRNA-mediated EphA2-reduced breast cancer cell lines were also used to elucidate the effect that EphA2 has on angiogenesis. *In vitro* studies measuring VEGF concentrations in the supernatant of cells were largely inconclusive. Preliminary studies done by members in my lab were able to show significant reduction in the VEGF levels of cells treated with EphA2-targeting siRNA, but I was unable to repeat this experiment to achieve the same level of significance (Figure 19). Furthermore, qRT-PCR analysis revealed no significant change in VEGF mRNA levels in MDA-MB-231 Luc and BT549 cells that had stably reduced levels of EphA2 in comparison to the EphA2 expressing cells. However, CD31 staining of tumor tissue sections of the mice with MDA-MB-231 Luc LV3 mammary fat pad tumors revealed a significant decrease in the microvessel density compared to the control tumors

that were also stained for CD31 (Figure 11). The conclusion to be drawn from this is that EphA2 may have an effect on the angiogenic capability of tumors, but it can not be assessed *in vitro* by way of VEGF analysis. The tumor environment *in vivo* is much different from the conditions in cell culture and this may be the cause of the seemingly contradictory data. EphA2's role in angiogenesis may not be direct and may involve signaling through other pathways such that simply reducing EphA2 levels would not be sufficient to see a significant decrease in VEGF. Studies done in ovarian cancer show that EphA2 overexpression highly correlated with angiogenesis *in vivo* (66). This relationship was not as clear in the metastatic breast cancer model, but that does not mean that EphA2 does not play a role in this process in breast cancer, rather, it requires further investigation.

In vivo investigation into the ability of stably reduced EphA2 breast cancer cells to develop metastases revealed that they are not as efficient at establishing lung metastases as their EphA2 overexpressing counterparts (Figure 12). These data suggest that not only is EphA2 important for breast cancer cell migration away from the primary tumor and into circulation, but it is also important in the ability of these circulating tumor cells to successfully establish secondary tumors elsewhere in the body. As previously discussed, EphA2 is known for its expression in late stage, aggressive breast cancers and these data further implicate that this overexpression is one of the mechanisms that gives breast cancer cells increased metastatic capabilities during breast cancer progression. Furthermore, tumor establishment using metastatic breast cancer cell lines with shRNA-mediated EphA2 reduction revealed that mammary fat pad tumors with reduced EphA2 expression showed decreased tumor growth (Figure 9). This trend was first shown using

the MDA-MB-231 Luc stable cell lines and this result was repeated in a second experiment. Athymic female nude mice were also injected in the mammary fat pad with the BT549 stable cell lines, but tumor growth was not observed in either of the BT549 cell lines (LVNC nor LV3) suggesting that more cells may need to be injected into the mammary fat pads for the cells to establish tumors. Subsequent Ki-67 and cleaved caspase 3 staining of the MDA-MB-231 Luc LVNC and LV3 tumor tissue sections collected from the above experiment did not reveal any differences in Ki-67 or cleaved caspase 3 staining in the LVNC mammary fat pad tumor tissue sections when compared to the LV3 mammary fat pad tumor tissue sections. The reason for decreased tumor growth in these tumors is not related to a decrease in cellular proliferation or an increase in cellular apoptosis, possibly due to the relatively modest growth differences in tumors. My results suggested that the EphA2 siRNA delivery to tumors using nanoparticles in the absence of chemotherapy was the more effective way to knockdown EphA2 receptor expression in established mammary fat pad tumors *in vivo* (Figure 21). However, this knockdown was modest when compared with the DOPC liposomes. The indication is that while there was a modest reduction in tumor growth observed after treatment of the mammary fat pad tumors with EphA2-targeting nanoparticles, the quantity of siRNA delivered was not sufficient to reduce EphA2 levels to the point where tumor growth was significantly impaired. It's important to note that delivery of EphA2 siRNA in DOPC liposomes has been shown to be effective in an ovarian cancer model after i.p. administration which contrasts with our systemic administration. My results indicate that increased doses and/or frequency of siRNA injections might lead to more conclusive results. Another consideration is that EphA2 expression is driven through the Ras-MAPK

pathway and the MDA-MB-231 Luc cells express an activating K-Ras mutation (77). A more effective approach to diminishing EphA2 receptor levels in this model system may involve targeting the EphA2 gene in addition to the Ras-MAPK pathway.

In summation, the data collected from this set of experiments that sought to elucidate the effect of EphA2 knockdown in a metastatic breast cancer model *in vitro* and *in vivo* has revealed that EphA2 does play a role in promoting the metastatic potential of breast cancer cells. My work has helped to establish that EphA2 seems to have the same function in cancer progression as it does during embryogenesis and mammary branching morphogenesis, where it aids in the migratory capability of cells. As much as I would like to have been able to propose a model for how EphA2 is functioning in the greater scheme of things, my work has been more important in confirming that EphA2 functions in breast cancer cells similar to how it functions in other cancer models where the mechanisms have already been investigated. Despite this progress, more research should be done to better understand the effect of EphA2 receptor depletion in metastatic breast cancer cells and tumors. It is possible that although EphA2 does assist in the cellular mechanisms that promote metastasis, therapeutically targeting it for the treatment of metastatic breast cancer may not confer a sufficient anti-tumoral effect as a single agent. Many molecular targeted therapies have been shown to be more effective when combined in standard therapies. Despite this, EphA2 is still an important receptor due to its robust expression in nearly all metastatic breast cancer cell lines. Since triple-negative breast cancers are largely defined by the molecular markers that they lack. Knowing that EphA2's expression is common in these cancers and that it is not highly expressed in other tissues makes it a possible molecular target for cytotoxic therapy homing (128, 129). Overall,

EphA2 is an important receptor tyrosine kinase in metastatic breast cancers and the unexplored mechanisms behind its modes of action could potentially have relevant biologic and therapeutic significance, some of which has been elucidated by the work done for this thesis.

Future Directions

The role of EphA2 in the metastatic capabilities of metastatic breast cancer cells should continue to be investigated in the breast cancer model. EphA2 has been shown to be a participant in other signaling pathways, such as FAK-mediated cellular interaction with the ECM in epithelial prostate cancer cells (60). EphA2's role in pathways such as these has yet to be elucidated and investigation into them may highly contribute to gaining a more complete understanding of EphA2's role not only in breast cancer, but in other cancer models as well. Furthermore, as discussed in the summary, it may be that EphA2's unique expression on cancerous epithelial cells is going to be more clinically beneficial than attempting to hinder the downstream signaling actions that result from EphA2 overexpression. For example, using the expression of EphA2 as a tumor homing mechanism for cytotoxic therapy against metastatic breast cancer might be worth exploring in future studies.

On a larger scale, the Eph receptors in general have been the target of drug developers (130). EphA2 has specifically been the target of drug companies like MedImmune which had an anti-EphA2 antibody program, but unfortunately, this project was discontinued for unspecified reasons after its acquisition by Astra-Zeneca in 2008 (130). Furthermore, Pfizer has a preclinical study of an anti-EphA2 tyrosine kinase inhibitor that is still ongoing (130). The attention given to EphA2 by drug companies signifies that it is an important potential therapeutic target not only for breast cancer patients, but for other cancer patients as well. However, development of anti-EphA2 therapies still has a long way to go and it is very possible that multiple anti-Eph

therapies will be needed in order to inhibit the tumor promoting effects of this receptor tyrosine kinase family.

Conclusions

EphA2 is overexpressed in the triple-negative, basal subtype of breast cancers and these breast cancers are highly aggressive and metastatic. EphA2 is a participant in the migratory ability of breast cancer cells, giving them increased metastatic ability. Furthermore, it appears that reduced levels of EphA2 causes a decrease in the MVD of mammary fat pad tumors, but the mechanism behind this observation is still unclear. Additionally, reduced levels of EphA2 were found to correlate with decreased tumor growth *in vivo* both after treatment of established mammary fat tumors with nanoparticles containing EphA2 siRNA, and also in mammary fat pad tumors that were established using EphA2-reduced breast cancer cells. While there is still much investigation that needs to be done, this work establishes that EphA2 is important in promoting the characteristics that allow breast cancer cells to metastasize.

References

1. Jemal, A., R. Siegel, J. Xu, and E. Ward. Cancer statistics, 2010. *CA Cancer J Clin* 60:277-300.
2. Vargo-Gogola, T., and J. M. Rosen. 2007. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer* 7:659-672.
3. Welch, D. R., P. S. Steeg, and C. W. Rinker-Schaeffer. 2000. Molecular biology of breast cancer metastasis. Genetic regulation of human breast carcinoma metastasis. *Breast Cancer Res* 2:408-416.
4. Bland, K. I., Copeland, E.M. , editor. 1991. *The Breast: Comprehensive Management of Benign and Malignant Diseases*. W. B. Saunders Company Philadelphia.
5. New York-Presbyterian, (2008) "Breast Anatomy".
<http://nyp.org/health/breast-anatomy.html> (November 30, 2008).
6. Adriance, M. C., J. L. Inman, O. W. Petersen, and M. J. Bissell. 2005. Myoepithelial cells: good fences make good neighbors. *Breast Cancer Res* 7:190-197.
7. Rakha, E. A., and I. O. Ellis. 2009. Triple-negative/basal-like breast cancer: review. *Pathology* 41:40-47.
8. Neve, R. M., K. Chin, J. Fridlyand, J. Yeh, F. L. Baehner, T. Fevr, L. Clark, N. Bayani, J. P. Coppe, F. Tong, T. Speed, P. T. Spellman, S. DeVries, A. Lapuk, N. J. Wang, W. L. Kuo, J. L. Stilwell, D. Pinkel, D. G. Albertson, F. M. Waldman, F. McCormick, R. B. Dickson, M. D. Johnson, M. Lippman, S. Ethier, A. Gazdar,

- and J. W. Gray. 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 10:515-527.
9. National Cancer Institute (2010) "What You Need to Know About Breast Cancer". <http://www.cancer.gov/cancertopics/wyntk/breast/page1> (October 15, 2009).
 10. Comet, B., B. Cutuli, F. Penault-Llorca, J. Bonnetterre, and Y. Belkacemi. 2009. [Male breast cancer: a review]. *Bull Cancer* 96:181-189.
 11. Lester, J. 2007. Breast cancer in 2007: incidence, risk assessment, and risk reduction strategies. *Clin J Oncol Nurs* 11:619-622.
 12. Davidson, N. E. 1992. Biology of breast cancer and its clinical implications. *Curr Opin Oncol* 4:1003-1009.
 13. Sorlie, T., C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, P. Eystein Lonning, and A. L. Borresen-Dale. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98:10869-10874.
 14. Manfrin, E., R. Mariotto, A. Remo, D. Reghellin, F. Falsirollo, D. Dalfior, P. Bricolo, E. Piazzola, and F. Bonetti. 2009. Benign breast lesions at risk of developing cancer--a challenging problem in breast cancer screening programs: five years' experience of the Breast Cancer Screening Program in Verona (1999-2004). *Cancer* 115:499-507.

15. Li, C. I., K. E. Malone, B. S. Saltzman, and J. R. Daling. 2006. Risk of invasive breast carcinoma among women diagnosed with ductal carcinoma in situ and lobular carcinoma in situ, 1988-2001. *Cancer* 106:2104-2112.
16. Frykberg, E. R. 1999. Lobular Carcinoma In Situ of the Breast. *Breast J* 5:296-303.
17. Robertson, F. M., M. Bondy, W. Yang, H. Yamauchi, S. Wiggins, S. Kamrudin, S. Krishnamurthy, H. Le-Petross, L. Bidaut, A. N. Player, S. H. Barsky, W. A. Woodward, T. Buchholz, A. Lucci, N. Ueno, and M. Cristofanilli. Inflammatory Breast Cancer: The Disease, the Biology, the Treatment. *CA Cancer J Clin*.
18. Perou, C. M., T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lonning, A. L. Borresen-Dale, P. O. Brown, and D. Botstein. 2000. Molecular portraits of human breast tumours. *Nature* 406:747-752.
19. Ishihara, A., H. Tsuda, K. Kitagawa, M. Yoneda, and T. Shiraishi. 2009. Morphological characteristics of basal-like subtype of breast carcinoma with special reference to cytopathological features. *Breast Cancer* 16:179-185.
20. Bertucci, F., P. Finetti, N. Cervera, E. Charafe-Jauffret, M. Buttarelli, J. Jacquemier, M. Chaffanet, D. Maraninchi, P. Viens, and D. Birnbaum. 2009. How different are luminal A and basal breast cancers? *Int J Cancer* 124:1338-1348.
21. Eeckhoute, J., E. K. Keeton, M. Lupien, S. A. Krum, J. S. Carroll, and M. Brown. 2007. Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. *Cancer Res* 67:6477-6483.

22. Loi, S. 2008. Molecular analysis of hormone receptor positive (luminal) breast cancers: what have we learnt? *Eur J Cancer* 44:2813-2818.
23. Slamon, D. J. 1987. Proto-oncogenes and human cancers. *N Engl J Med* 317:955-957.
24. Ferretti, G., A. Felici, P. Papaldo, A. Fabi, and F. Cignetti. 2007. HER2/neu role in breast cancer: from a prognostic foe to a predictive friend. *Curr Opin Obstet Gynecol* 19:56-62.
25. Dean-Colomb, W., and F. J. Esteva. 2008. Her2-positive breast cancer: herceptin and beyond. *Eur J Cancer* 44:2806-2812.
26. Rakha, E. A., J. S. Reis-Filho, and I. O. Ellis. 2008. Basal-like breast cancer: a critical review. *J Clin Oncol* 26:2568-2581.
27. Honeth, G., P. O. Bendahl, M. Ringner, L. H. Saal, S. K. Gruvberger-Saal, K. Lovgren, D. Grabau, M. Ferno, A. Borg, and C. Hegardt. 2008. The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res* 10:R53.
28. Singletary, S. E., and J. L. Connolly. 2006. Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual. *CA Cancer J Clin* 56:37-47; quiz 50-31.
29. Acharya, C. R., D. S. Hsu, C. K. Anders, A. Anguiano, K. H. Salter, K. S. Walters, R. C. Redman, S. A. Tuchman, C. A. Moylan, S. Mukherjee, W. T. Barry, H. K. Dressman, G. S. Ginsburg, K. P. Marcom, K. S. Garman, G. H. Lyman, J. R. Nevins, and A. Potti. 2008. Gene expression signatures,

- clinicopathological features, and individualized therapy in breast cancer. *JAMA* 299:1574-1587.
30. Hunt, K. K., Robb, G.L., Strom, E.A., Ueno, N.T., editor. 2008. *Breast Cancer*. Springer, Houston.
 31. Buchholz, T. A. 2009. Radiation therapy for early-stage breast cancer after breast-conserving surgery. *N Engl J Med* 360:63-70.
 32. Hortobagyi, G. N. 1998. Treatment of breast cancer. *N Engl J Med* 339:974-984.
 33. Cleator, S., W. Heller, and R. C. Coombes. 2007. Triple-negative breast cancer: therapeutic options. *Lancet Oncol* 8:235-244.
 34. Pasquale, E. B. 1997. The Eph family of receptors. *Curr Opin Cell Biol* 9:608-615.
 35. Hirai, H., Y. Maru, K. Hagiwara, J. Nishida, and F. Takaku. 1987. A novel putative tyrosine kinase receptor encoded by the eph gene. *Science* 238:1717-1720.
 36. Pasquale, E. B. 2005. Eph receptor signalling casts a wide net on cell behaviour. *Nat Rev Mol Cell Biol* 6:462-475.
 37. Davis, S., N. W. Gale, T. H. Aldrich, P. C. Maisonpierre, V. Lhotak, T. Pawson, M. Goldfarb, and G. D. Yancopoulos. 1994. Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* 266:816-819.
 38. Flanagan, J. G., and P. Vanderhaeghen. 1998. The ephrins and Eph receptors in neural development. *Annu Rev Neurosci* 21:309-345.

39. Kullander, K., and R. Klein. 2002. Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* 3:475-486.
40. Ogawa, K., R. Pasqualini, R. A. Lindberg, R. Kain, A. L. Freeman, and E. B. Pasquale. 2000. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. *Oncogene* 19:6043-6052.
41. Lindberg, R. A., and T. Hunter. 1990. cDNA cloning and characterization of eck, an epithelial cell receptor protein-tyrosine kinase in the eph/elk family of protein kinases. *Mol Cell Biol* 10:6316-6324.
42. Hafner, C., G. Schmitz, S. Meyer, F. Bataille, P. Hau, T. Langmann, W. Dietmaier, M. Landthaler, and T. Vogt. 2004. Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. *Clin Chem* 50:490-499.
43. Kouros-Mehr, H., and Z. Werb. 2006. Candidate regulators of mammary branching morphogenesis identified by genome-wide transcript analysis. *Dev Dyn* 235:3404-3412.
44. Wykosky, J., and W. Debinski. 2008. The EphA2 receptor and ephrinA1 ligand in solid tumors: function and therapeutic targeting. *Mol Cancer Res* 6:1795-1806.
45. Pandey, A., H. Duan, and V. M. Dixit. 1995. Characterization of a novel Src-like adapter protein that associates with the Eck receptor tyrosine kinase. *J Biol Chem* 270:19201-19204.
46. Cheng, N., D. M. Brantley, and J. Chen. 2002. The ephrins and Eph receptors in angiogenesis. *Cytokine Growth Factor Rev* 13:75-85.

47. Holder, N., and R. Klein. 1999. Eph receptors and ephrins: effectors of morphogenesis. *Development* 126:2033-2044.
48. Ganju, P., K. Shigemoto, J. Brennan, A. Entwistle, and A. D. Reith. 1994. The Eck receptor tyrosine kinase is implicated in pattern formation during gastrulation, hindbrain segmentation and limb development. *Oncogene* 9:1613-1624.
49. Goldman-Wohl, D., C. Greenfield, R. Haimov-Kochman, I. Ariel, E. Y. Anteby, D. Hochner-Celnikier, M. Farhat, and S. Yagel. 2004. Eph and ephrin expression in normal placental development and preeclampsia. *Placenta* 25:623-630.
50. Kinch, M. S., and K. Carles-Kinch. 2003. Overexpression and functional alterations of the EphA2 tyrosine kinase in cancer. *Clin Exp Metastasis* 20:59-68.
51. Miao, H., C. H. Nickel, L. G. Cantley, L. A. Bruggeman, L. N. Bennardo, and B. Wang. 2003. EphA kinase activation regulates HGF-induced epithelial branching morphogenesis. *J Cell Biol* 162:1281-1292.
52. Andres, A. C., G. Zuercher, V. Djonov, M. Flueck, and A. Ziemiecki. 1995. Protein tyrosine kinase expression during the estrous cycle and carcinogenesis of the mammary gland. *Int J Cancer* 63:288-296.
53. Zelinski, D. P., N. D. Zantek, J. C. Stewart, A. R. Irizarry, and M. S. Kinch. 2001. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. *Cancer Res* 61:2301-2306.
54. Andres, A. C., H. H. Reid, G. Zurcher, R. J. Blaschke, D. Albrecht, and A. Ziemiecki. 1994. Expression of two novel eph-related receptor protein tyrosine

- kinases in mammary gland development and carcinogenesis. *Oncogene* 9:1461-1467.
55. Himanen, J. P., K. R. Rajashankar, M. Lackmann, C. A. Cowan, M. Henkemeyer, and D. B. Nikolov. 2001. Crystal structure of an Eph receptor-ephrin complex. *Nature* 414:933-938.
 56. Walker-Daniels, J., A. R. Hess, M. J. Hendrix, and M. S. Kinch. 2003. Differential regulation of EphA2 in normal and malignant cells. *Am J Pathol* 162:1037-1042.
 57. Pandey, A., D. F. Lazar, A. R. Saltiel, and V. M. Dixit. 1994. Activation of the Eck receptor protein tyrosine kinase stimulates phosphatidylinositol 3-kinase activity. *J Biol Chem* 269:30154-30157.
 58. Pratt, R. L., and M. S. Kinch. 2002. Activation of the EphA2 tyrosine kinase stimulates the MAP/ERK kinase signaling cascade. *Oncogene* 21:7690-7699.
 59. Zantek, N. D., M. Azimi, M. Fedor-Chaiken, B. Wang, R. Brackenbury, and M. S. Kinch. 1999. E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. *Cell Growth Differ* 10:629-638.
 60. Miao, H., E. Burnett, M. Kinch, E. Simon, and B. Wang. 2000. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat Cell Biol* 2:62-69.
 61. Ireton, R. C., and J. Chen. 2005. EphA2 receptor tyrosine kinase as a promising target for cancer therapeutics. *Curr Cancer Drug Targets* 5:149-157.
 62. Noblitt, L. W., D. S. Bangari, S. Shukla, D. W. Knapp, S. Mohammed, M. S. Kinch, and S. K. Mittal. 2004. Decreased tumorigenic potential of EphA2-

- overexpressing breast cancer cells following treatment with adenoviral vectors that express EphrinA1. *Cancer Gene Ther* 11:757-766.
63. Zeng, G., Z. Hu, M. S. Kinch, C. X. Pan, D. A. Flockhart, C. Kao, T. A. Gardner, S. Zhang, L. Li, L. A. Baldrige, M. O. Koch, T. M. Ulbright, J. N. Eble, and L. Cheng. 2003. High-level expression of EphA2 receptor tyrosine kinase in prostatic intraepithelial neoplasia. *Am J Pathol* 163:2271-2276.
64. Duxbury, M. S., H. Ito, M. J. Zinner, S. W. Ashley, and E. E. Whang. 2004. EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma. *Oncogene* 23:1448-1456.
65. Thaker, P. H., M. Deavers, J. Celestino, A. Thornton, M. S. Fletcher, C. N. Landen, M. S. Kinch, P. A. Kiener, and A. K. Sood. 2004. EphA2 expression is associated with aggressive features in ovarian carcinoma. *Clin Cancer Res* 10:5145-5150.
66. Lin, Y. G., L. Y. Han, A. A. Kamat, W. M. Merritt, C. N. Landen, M. T. Deavers, M. S. Fletcher, D. L. Urbauer, M. S. Kinch, and A. K. Sood. 2007. EphA2 overexpression is associated with angiogenesis in ovarian cancer. *Cancer* 109:332-340.
67. Hanahan, D., and R. A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100:57-70.
68. Brannan, J. M., W. Dong, L. Prudkin, C. Behrens, R. Lotan, B. N. Bekele, I. Wistuba, and F. M. Johnson. 2009. Expression of the receptor tyrosine kinase EphA2 is increased in smokers and predicts poor survival in non-small cell lung cancer. *Clin Cancer Res* 15:4423-4430.

69. Miyazaki, T., H. Kato, M. Fukuchi, M. Nakajima, and H. Kuwano. 2003. EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. *Int J Cancer* 103:657-663.
70. Easty, D. J., S. P. Hill, M. Y. Hsu, M. E. Fallowfield, V. A. Florenes, M. Herlyn, and D. C. Bennett. 1999. Up-regulation of ephrin-A1 during melanoma progression. *Int J Cancer* 84:494-501.
71. Saito, T., N. Masuda, T. Miyazaki, K. Kanoh, H. Suzuki, T. Shimura, T. Asao, and H. Kuwano. 2004. Expression of EphA2 and E-cadherin in colorectal cancer: correlation with cancer metastasis. *Oncol Rep* 11:605-611.
72. Wu, D., Z. Suo, G. B. Kristensen, S. Li, G. Troen, R. Holm, and J. M. Nesland. 2004. Prognostic value of EphA2 and EphrinA-1 in squamous cell cervical carcinoma. *Gynecol Oncol* 94:312-319.
73. Tatsumi, T., C. J. Herrem, W. C. Olson, J. H. Finke, R. M. Bukowski, M. S. Kinch, E. Ranieri, and W. J. Storkus. 2003. Disease stage variation in CD4+ and CD8+ T-cell reactivity to the receptor tyrosine kinase EphA2 in patients with renal cell carcinoma. *Cancer Res* 63:4481-4489.
74. Dickson, R. B., E. W. Thompson, and M. E. Lippman. 1989. Hormones and breast cancer in vitro. *Hum Cell* 2:219-230.
75. Zelinski, D. P., N. D. Zantek, J. Walker-Daniels, M. A. Peters, E. J. Taparowsky, and M. S. Kinch. 2002. Estrogen and Myc negatively regulate expression of the EphA2 tyrosine kinase. *J Cell Biochem* 85:714-720.
76. Dubik, D., P. H. Watson, M. Venditti, and R. P. Shiu. 1996. Nuclear oncogenes in breast cancer. *Cancer Treat Res* 83:171-189.

77. Macrae, M., R. M. Neve, P. Rodriguez-Viciana, C. Haqq, J. Yeh, C. Chen, J. W. Gray, and F. McCormick. 2005. A conditional feedback loop regulates Ras activity through EphA2. *Cancer Cell* 8:111-118.
78. Kozma, S. C., M. E. Bogaard, K. Buser, S. M. Saurer, J. L. Bos, B. Groner, and N. E. Hynes. 1987. The human c-Kirsten ras gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231. *Nucleic Acids Res* 15:5963-5971.
79. Wilhelm, S. M., C. Carter, L. Tang, D. Wilkie, A. McNabola, H. Rong, C. Chen, X. Zhang, P. Vincent, M. McHugh, Y. Cao, J. Shujath, S. Gawlak, D. Eveleigh, B. Rowley, L. Liu, L. Adnane, M. Lynch, D. Auclair, I. Taylor, R. Gedrich, A. Voznesensky, B. Riedl, L. E. Post, G. Bollag, and P. A. Trail. 2004. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 64:7099-7109.
80. Wijnhoven, B. P., W. N. Dinjens, and M. Pignatelli. 2000. E-cadherin-catenin cell-cell adhesion complex and human cancer. *Br J Surg* 87:992-1005.
81. Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris. 1991. p53 mutations in human cancers. *Science* 253:49-53.
82. Levine, A. J. 1997. p53, the cellular gatekeeper for growth and division. *Cell* 88:323-331.
83. Dohn, M., J. Jiang, and X. Chen. 2001. Receptor tyrosine kinase EphA2 is regulated by p53-family proteins and induces apoptosis. *Oncogene* 20:6503-6515.

84. Kikawa, K. D., D. R. Vidale, R. L. Van Etten, and M. S. Kinch. 2002. Regulation of the EphA2 kinase by the low molecular weight tyrosine phosphatase induces transformation. *J Biol Chem* 277:39274-39279.
85. Talmadge, J. E., and I. J. Fidler. AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res* 70:5649-5669.
86. Nicolson, G. L. 1988. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev* 7:143-188.
87. Fidler, I. J. 2002. The organ microenvironment and cancer metastasis. *Differentiation* 70:498-505.
88. Poste, G., and I. J. Fidler. 1980. The pathogenesis of cancer metastasis. *Nature* 283:139-146.
89. Fidler, I. J. 1970. Metastasis: quantitative analysis of distribution and fate of tumor embolilabeled with ¹²⁵I-5-iodo-2'-deoxyuridine. *J Natl Cancer Inst* 45:773-782.
90. Butler, T. P., and P. M. Gullino. 1975. Quantitation of cell shedding into efferent blood of mammary adenocarcinoma. *Cancer Res* 35:512-516.
91. Fidler, I. J. 1975. Biological behavior of malignant melanoma cells correlated to their survival in vivo. *Cancer Res* 35:218-224.
92. World Health Organization, (2004) "Global Burden of Disease".
http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html (2004).

93. Chambers, A. F., G. N. Naumov, S. A. Vantyghem, and A. B. Tuck. 2000. Molecular biology of breast cancer metastasis. Clinical implications of experimental studies on metastatic inefficiency. *Breast Cancer Res* 2:400-407.
94. ATCC, (2011) "Product Search: MDA-MB-231, BT549, HCC1954". <http://www.atcc.org/> (2011).
95. Landen, C. N., Jr., A. Chavez-Reyes, C. Bucana, R. Schmandt, M. T. Deavers, G. Lopez-Berestein, and A. K. Sood. 2005. Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. *Cancer Res* 65:6910-6918.
96. Zhang, C., J. T. Newsome, R. Mewani, J. Pei, P. C. Gokhale, and U. N. Kasid. 2009. Systemic delivery and pre-clinical evaluation of nanoparticles containing antisense oligonucleotides and siRNAs. *Methods Mol Biol* 480:65-83.
97. Rao, D. D., J. S. Vorhies, N. Senzer, and J. Nemunaitis. 2009. siRNA vs. shRNA: similarities and differences. *Adv Drug Deliv Rev* 61:746-759.
98. Nguyen, T., E. M. Menocal, J. Harborth, and J. H. Fruehauf. 2008. RNAi therapeutics: an update on delivery. *Curr Opin Mol Ther* 10:158-167.
99. Freedman, V. H., and S. I. Shin. 1974. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* 3:355-359.
100. Larsen, A. B., M. W. Pedersen, M. T. Stockhausen, M. V. Grandal, B. van Deurs, and H. S. Poulsen. 2007. Activation of the EGFR gene target EphA2 inhibits epidermal growth factor-induced cancer cell motility. *Mol Cancer Res* 5:283-293.
101. Larsen, A. B., M. T. Stockhausen, and H. S. Poulsen. Cell adhesion and EGFR activation regulate EphA2 expression in cancer. *Cell Signal* 22:636-644.

102. Miao, H., D. Q. Li, A. Mukherjee, H. Guo, A. Petty, J. Cutter, J. P. Basilion, J. Sedor, J. Wu, D. Danielpour, A. E. Sloan, M. L. Cohen, and B. Wang. 2009. EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. *Cancer Cell* 16:9-20.
103. Hiramoto-Yamaki, N., S. Takeuchi, S. Ueda, K. Harada, S. Fujimoto, M. Negishi, and H. Katoh. Ephexin4 and EphA2 mediate cell migration through a RhoG-dependent mechanism. *J Cell Biol* 190:461-477.
104. Frisch, S. M., and R. A. Screaton. 2001. Anoikis mechanisms. *Curr Opin Cell Biol* 13:555-562.
105. Rundhaug, J. E. 2003. Matrix metalloproteinases, angiogenesis, and cancer: commentary re: A. C. Lockhart et al., Reduction of wound angiogenesis in patients treated with BMS-275291, a broad spectrum matrix metalloproteinase inhibitor. *Clin. Cancer Res.*, 9: 00-00, 2003. *Clin Cancer Res* 9:551-554.
106. Vaught, D., D. M. Brantley-Sieders, and J. Chen. 2008. Eph receptors in breast cancer: roles in tumor promotion and tumor suppression. *Breast Cancer Res* 10:217.
107. Landen, C. N., W. M. Merritt, L. S. Mangala, A. M. Sanguino, C. Bucana, C. Lu, Y. G. Lin, L. Y. Han, A. A. Kamat, R. Schmandt, R. L. Coleman, D. M. Gershenson, G. Lopez-Berestein, and A. K. Sood. 2006. Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer. *Cancer Biol Ther* 5:1708-1713.

108. Lu, M., K. D. Miller, Y. Gokmen-Polar, M. H. Jeng, and M. S. Kinch. 2003. EphA2 overexpression decreases estrogen dependence and tamoxifen sensitivity. *Cancer Res* 63:3425-3429.
109. Scherr, M., M. A. Morgan, and M. Eder. 2003. Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr Med Chem* 10:245-256.
110. Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494-498.
111. Price, J. T., T. Tiganis, A. Agarwal, D. Djakiew, and E. W. Thompson. 1999. Epidermal growth factor promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3'-kinase and phospholipase C-dependent mechanism. *Cancer Res* 59:5475-5478.
112. Carmeliet, P. 2003. Angiogenesis in health and disease. *Nat Med* 9:653-660.
113. Brantley-Sieders, D. M., W. B. Fang, D. J. Hicks, G. Zhuang, Y. Shyr, and J. Chen. 2005. Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression. *FASEB J* 19:1884-1886.
114. Adams, R. H., and R. Klein. 2000. Eph receptors and ephrin ligands. essential mediators of vascular development. *Trends Cardiovasc Med* 10:183-188.
115. Brantley-Sieders, D. M., J. Caughron, D. Hicks, A. Pozzi, J. C. Ruiz, and J. Chen. 2004. EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation. *J Cell Sci* 117:2037-2049.

116. Hannon, G. J., and J. J. Rossi. 2004. Unlocking the potential of the human genome with RNA interference. *Nature* 431:371-378.
117. Seo, S. H., H. D. Han, K. H. Noh, T. W. Kim, and S. W. Son. 2009. Chitosan hydrogel containing GMCSF and a cancer drug exerts synergistic anti-tumor effects via the induction of CD8+ T cell-mediated anti-tumor immunity. *Clin Exp Metastasis* 26:179-187.
118. Han, H. D., L. S. Mangala, J. W. Lee, M. M. Shahzad, H. S. Kim, D. Shen, E. J. Nam, E. M. Mora, R. L. Stone, C. Lu, S. J. Lee, J. W. Roh, A. M. Nick, G. Lopez-Berestein, and A. K. Sood. Targeted gene silencing using RGD-labeled chitosan nanoparticles. *Clin Cancer Res* 16:3910-3922.
119. Villares, G. J., M. Zigler, H. Wang, V. O. Melnikova, H. Wu, R. Friedman, M. C. Leslie, P. E. Vivas-Mejia, G. Lopez-Berestein, A. K. Sood, and M. Bar-Eli. 2008. Targeting melanoma growth and metastasis with systemic delivery of liposome-incorporated protease-activated receptor-1 small interfering RNA. *Cancer Res* 68:9078-9086.
120. Pedersen, M. W., V. Tkach, N. Pedersen, V. Berezin, and H. S. Poulsen. 2004. Expression of a naturally occurring constitutively active variant of the epidermal growth factor receptor in mouse fibroblasts increases motility. *Int J Cancer* 108:643-653.
121. Taetle, R., J. M. Honeysett, and L. L. Houston. 1988. Effects of anti-epidermal growth factor (EGF) receptor antibodies and an anti-EGF receptor recombinant-ricin A chain immunoconjugate on growth of human cells. *J Natl Cancer Inst* 80:1053-1059.

122. Arteaga, C. L., S. D. Hurd, T. C. Dugger, A. R. Winnier, and J. B. Robertson. 1994. Epidermal growth factor receptors in human breast carcinoma cells: a potential selective target for transforming growth factor alpha-Pseudomonas exotoxin 40 fusion protein. *Cancer Res* 54:4703-4709.
123. Hollestelle, A., J. H. Nagel, M. Smid, S. Lam, F. Elstrodt, M. Wasielewski, S. S. Ng, P. J. French, J. K. Peeters, M. J. Rozendaal, M. Riaz, D. G. Koopman, T. L. Ten Hagen, B. H. de Leeuw, E. C. Zwarthoff, A. Teunisse, P. J. van der Spek, J. G. Klijn, W. N. Dinjens, S. P. Ethier, H. Clevers, A. G. Jochemsen, M. A. den Bakker, J. A. Foekens, J. W. Martens, and M. Schutte. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. *Breast Cancer Res Treat* 121:53-64.
124. Lin, P., S. Sankar, S. Shan, M. W. Dewhirst, P. J. Polverini, T. Q. Quinn, and K. G. Peters. 1998. Inhibition of tumor growth by targeting tumor endothelium using a soluble vascular endothelial growth factor receptor. *Cell Growth Differ* 9:49-58.
125. He, M., J. Rosen, D. Mangiameli, and S. K. Libutti. 2007. Cancer development and progression. *Adv Exp Med Biol* 593:117-133.
126. Margaryan, N. V., L. Strizzi, D. E. Abbott, E. A. Seftor, M. S. Rao, M. J. Hendrix, and A. R. Hess. 2009. EphA2 as a promoter of melanoma tumorigenicity. *Cancer Biol Ther* 8:279-288.
127. Sporn, M. B. 1996. The war on cancer. *Lancet* 347:1377-1381.
128. Lee, J. W., H. D. Han, M. M. Shahzad, S. W. Kim, L. S. Mangala, A. M. Nick, C. Lu, R. R. Langle, R. Schmandt, H. S. Kim, S. Mao, J. Gooya, C. Fazenbaker, D. Jackson, D. A. Tice, C. N. Landen, R. L. Coleman, and A. K. Sood. 2009. EphA2

- immunoconjugate as molecularly targeted chemotherapy for ovarian carcinoma. *J Natl Cancer Inst* 101:1193-1205.
129. Jackson, D., J. Gooya, S. Mao, K. Kinneer, L. Xu, M. Camara, C. Fazenbaker, R. Fleming, S. Swamynathan, D. Meyer, P. D. Senter, C. Gao, H. Wu, M. Kinch, S. Coats, P. A. Kiener, and D. A. Tice. 2008. A human antibody-drug conjugate targeting EphA2 inhibits tumor growth in vivo. *Cancer Res* 68:9367-9374.
130. Garber, K. Of Ephs and ephrins: companies target guidance molecules in cancer. *J Natl Cancer Inst* 102:1692-1694.

Vitae

Stephanie Ann Erzinger was born on June 28, 1986 in Copenhagen, Denmark, is the daughter of Frank Bradley Erzinger and Kathy Ann Erzinger and is the sister to Steven Bradley Erzinger, Scott Christopher Erzinger, and Alison Lee Erzinger. After graduating from M.B. Lamar High School in Houston, Texas, Stephanie enrolled at Louisiana State University in Baton Rouge, Louisiana. A Bachelor of Science degree in Biological Sciences was granted to her in December of 2007. After receiving her degree, Stephanie traveled to Buenos Aires, Argentina where she studied Spanish and taught English. In August of 2008, Stephanie enrolled in the M.S. program at the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. In January 2009, Stephanie joined the laboratory of Dr. Janet E. Price, where she studied the effects of silencing EphA2 in metastatic breast cancers.