EPHA2 RECEPTOR TYROSINE KINASE IN MAMMARY GLAND DEVELOPMENT AND BREAST CANCER INDUCED OSTEOLYSIS

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

May 2011

Nashville, Tennessee

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ORIGINAL PUBLICATIONS

1. Regulation of heart valve morphogenesis by Eph receptor ligand, ephrin-A1. Frieden LA, Townsend TA, Vaught DB, Delaughter DM, Hwang Y, Barnett JV, Chen J. Dev Dyn. 2010 Oct 19.

2. Tie2 signaling regulates osteoclastogenesis and osteolytic bone invasion of breast cancer. Min Y, Ren X, Vaught DB, Chen J, Donnelly E, Lynch CC, Lin PC. Cancer Res. 2010 Apr 1;70(7):2819-28.

3. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Zhuang G, Brantley-Sieders DM, Vaught D, Yu J, Xie L, Wells S, Jackson D, Muraoka-Cook R, Arteaga C, Chen J. Cancer Res. 2010 Jan 1;70(1):299-308.

4. Host deficiency in Vav2/3 guanine nucleotide exchange factors impairs tumor growth, survival, and angiogenesis in vivo. Brantley-Sieders DM, Zhuang G, Vaught D, Freeman T, Hwang Y, Hicks D, Chen J. Mol Cancer Res. 2009 May;7(5):615-23.

5. Regulation of mammary gland branching morphogenesis by EphA2 receptor tyrosine kinase. Vaught D, Chen J, Brantley-Sieders DM. Mol Biol Cell. 2009 May;20(10):2572-81.

6. Eph receptor in breast cancer: roles in tumor promotion and tumor suppression. Vaught D, Brantley-Sieders DM, Chen J. Breast Cancer Res. 2008;10(6):217.

This is dedicated to my wife Elizabeth,
for her unconditional love, support and encouragement throughout this process.

In Memorium:
To my father-in-law Dr. Louis Moore

ACKNOWLEDGEMENTS

I owe tremendous gratitude to all those that have supported and encouraged me during my studies here at Vanderbilt. I would like to first thank my mentor, Dr. Jin Chen. This work could not have been completed without her guidance and insight. I have learned a tremendous amount from her as she has shaped me into a knowledgeable, driven, and focused scientist. I would also like to express my deepest appreciation to my thesis committee: Dr. Lynn Matrisian (chair), Dr. Pampee Young and Dr. Charles Lin. Their advice and critiques have made me a stronger scientist and have taught me how to critically analyze problems, evaluate results and present results effectively. I would also like to thank those who supported me financially during my graduate training, specifically to Dr. Carlos Arteaga with his breast cancer training grant 5T32 CA078136-10, and the Department of Defense with their predoctoral fellowship W81XWH-08-1-0249.

I would also like to thank the members of the Chen lab: Dana Brantley-Sieders, Donna Hicks, Wei Fang, Sonja Hunter, Guanglei Zhuang, Yoonha Hwang, Leslie Frieden, Meghana Rao, Katherine Amato, Charlene Dunaway, Krishna Sarma, and Wenqiang Song, as they have proved instrumental in my graduate work. Each member has played an integral role in my PhD whether it was through a simple “math check,” assisting with animals, or helping with the planning of an experiment.

A number of people outside of Dr. Chen’s lab proved critical in the development of my projects. Many of you shared valuable technical or

intellectual guidance that aided my studies as I ventured into an area of study outside the primary breast cancer focus of our lab. To you, Drs. Greg Mundy, Conor Lynch, James Edwards, Claire Edwards, Justin Cates, and Rebecca Cook, I say thank you.

A special thank you is also in order for Dr. Tom Aune's, Dr. Linda Sealy's, and Dr. Rebecca Cook's lab who have equipped me with reagents, equipment, and protocols. To Mel Henderson I owe a special thank you for assisting with animal experiments when I needed a helping hand. Your delicious food was also a welcome blessing. To Allison Atwood, Susan Yanni, Alisha Russell, Kim Boelte, Joe Deweese, Britney Grayson and Nyk Reed thank you for listening and offering words of encouragement, as well as entertaining me with frank and honest discussions regarding science, sports, and/or life in general. Thank you.

My studies could not and would not be completed without the support of friends and family. To Paul and Tracy Barth, Jared and Bec Monger, Jeremy and Karrisa Porter, David and Nicole Hannah, Shawn and April DeMers, Travis and Anita Simpson and Jennifer Moore, thank you for listening when you didn't understand, always keeping me humble, encouraging me daily, but most of all for hitting your knees for Elizabeth and me throughout the struggles of science. To my family and my mother-in-law, I thank you for your endless love, interest and support. I am thankful beyond words for the support you have offered. Your continuous interest in how the mice are doing was always welcomed.

Finally, I must thank my wife Elizabeth. Without her I wouldn't be at Vanderbilt and wouldn't have half of the success I enjoy. She tirelessly pushed

me to work smarter rather than harder and to be the best that I could be. She dealt with the long hours, the weekends, the tardy arrivals, and patiently listened (or glazed over) when I described my projects. She has learned as much as I during this time, especially in regards to timing, as she quickly learned when a scientist says “ten more minutes” they really mean 45 more minutes. All I can say is thank you for your patience and understanding. It is not easy to be married to a scientist, but despite the many scientific setbacks, she has always been my biggest supporter. I love you.

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ABBREVIATIONS

APC – Adenomatous Polyposis Coli

BMM – Bone Marrow Macrophage

BMP – Bone Morphogenic Protein

DAB – Diaminobenzidine Tetrahydrochloride

DMEM – Dulbecco's Modified Eagle's Medium

DOPC – 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine

ECL – Enhanced Chemiluminscence

ECM – Extraceullar Matrix

EDTA – Ethylenediaminetetraacetic Acid

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

ELISA – Enzyme Linked Immunosorbent Assay

EMT – Epithelial to Mesenchymal Transition

Eph – Erythopoietin Producing Hepatoma

Ephrin – Eph Family Receptor Interacting Proteins

ErbB2 – Erythroblastic Leukemia Viral Oncogene Homolog 2

ERK – Extracellular Signal-Regulated Kinase

FBS – Fetal Bovine Serum

FGF – Fibroblast Growth Factor

FGFR – Fibroblast Growth Factor Receptor

GBM – Glioblastoma Multiforme

GPI – Glycosylphosphatidlinositol

GST – Glutathione-S-Transferase

HCl – Hydrochloric Acid

HER2 – Human Epidermal Growth Factor Receptor 2

IGF – Insulin Growth Factor

IHC – Immunohistochemistry

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-11 – Interleukin 11

MAPK – Mitogen Activated Protein Kinase

MCSF- Macrophage Colony Stimulating Factor

MEK – Mitogen-Activated Protein Kinase Kinase

MeOH – Methanol

MMTV – Mouse Mammary Tumor Virus

MMP – Matrix Metalloproteinases

MSC – Mesenchymal Stem Cell

OPG – Osteopogerin

OPN – Osteopontin

PAGE – Polyacrylamide Gel Electrophoresis

PCNA – Proliferating Cell Nuclear Antigen

PCR – Polymerase Chain Reaction

PDZ – PSD95/Dlg/ZO1 Motif

PIP3 – Phosphatidylinositol 3,4,5-trisphosphate

PI3K – phosphatidylinositol 3 kinase

PMEC – Primary Mammary Epithelial Cells

PTHrP – Parathyroid Hormone Related Protein

RANKL – Receptor Activator of Nuclear Factor-κB Ligand

RTK – Receptor Tyrosine Kinase

SAM - Sterile-α-Motif

SDS – Sodium Dodecyl Sulfate

SEM – Standard Error of the Mean

SH2 – Src Homology 2

SHIP2 – Src Homology 2 Domain-Containing Phosphoinositide 5-Phosphatase 2

SHP2 – Src Homology 2-Containing Tyrosine Phosphatase 2

siRNA – Small Interferring RNA

TEB – Terminal End Bud

TED – Terminal End Duct

TGFβ – Transforming Growth Factor β

TMB – Tetramethylbenzidine

TUNEL – Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling

VEGF – Vascular Endothelial Growth Factor

vWF – von Willebrand Factor

CHAPTER I

INTRODUCTION

Overview

Since its discovery two decades ago, the Eph family of receptor tyrosine kinases (RTKs) has been implicated in an increasing number of physiological and pathological processes in various cell types and organs. Recent genome-wide studies in human cancer revealed that expression of Eph receptors are often dysregulated in many types of cancer and somatic mutations in tumors have been discovered in nearly all Eph receptors. However, despite the clinical relevance of Eph receptor tyrosine kinases in human cancer, their precise roles in cancer are not well understood. In breast cancer, EphA2 receptor is overexpressed in over 60% of tumor samples and high levels of EphA2 expression are associated with poor patient prognosis. Although roles of EphA2 in tumor growth and metastasis have been reported in a number of tumor models, the function of EphA2 in mammary gland development and breast cancer-induced osteolysis in bone metastasis has not been investigated. In my thesis work, we discovered that the EphA2 receptor plays a critical role in normal mammary epithelial proliferation and branching through regulating RhoA activity. Furthermore, we found that breast cancer cell-induced osteolysis is dependent on EphA2 function. Our genetic, molecular, and pharmacologic approaches demonstrate that signaling through class A Eph RTKs, particularly EphA2, is

critical for normal breast epithelial growth and morphogenesis, as well as tumor-induced osteolysis, providing a sound rationale for targeting EphA2 for new breast cancer therapies.

The Eph Receptors and Ephrin Ligands

The Eph receptors comprise the largest family of receptor tyrosine kinases discovered in the human genome, consisting of 15 receptors and nine ligands (Table 1) [1]. The family is subdivided into two subclasses based on sequence homology, binding affinity, and structure of the ephrin ligand. The A-subclass of receptors (EphA1-EphA10) bind to ligands tethered to the cell membrane by a glycosylphosphatidlinositol (GPI) anchor (ephrinA1-ephrinA6), while the B-subclass (EphB1-EphB4, EphB6) bind to ligands containing a transmembrane domain followed by a short cytoplasmic region (ephrinB1-ephrinB3) (Figure 1).

The extracellular portion of Eph receptors contain a highly conserved N-terminal ephrin-binding domain, a cysteine-rich region (including an epidermal growth factor-like motif) and two fibronectin type-III repeats. The Eph receptor cytoplasmic side is composed of a juxtamembrane segment, a classical protein tyrosine kinase domain, a sterile-α-motif (SAM) domain and a PSD95/Dlg/ZO1 (PDZ)-binding motif (Figure 1).

Eph Signaling

Engagement of Eph RTKs with ephrin ligands results in dimerization or oligomerization of receptor complexes on the cell membrane leading to

autophosphorylation of tyrosine residues throughout the intracellular portion of the receptor. Phosphorylation of the receptor's tyrosine amino acids within the juxtamembrane region releases the structural inhibition of the tyrosine residues in the kinase region permitting docking sites for downstream signaling molecules [3, 4].

Of the multiple signaling pathways affecting cell behavior mediated by Ephs and ephrins, the most commonly affected in breast cancer are Ras/MAPK, PI3K/AKT, and the small GTPases Rho/Rac/cdc42. Several publications have reported the ability of EphA2 to activate Ras/MAPK signaling [5-7]. However, other reports have demonstrated EphA2 signaling can attenuate Ras/MAPK signaling highlighting a controversy that will be discussed in more detail below [8]. The involvement of the small GTPases with Eph receptors, especially Rho, is detailed in Noren and Pasquale ([9]. Similarly, studies from our lab suggest a role of crosstalk by EphA2 and ErbB2 receptors to enhance RhoA activity [5] as well as the ability of EphA2 stimulation and phosphorylation to activate RhoA and affect cell migration [10, 11]. Eph receptor signaling with PI3K/AKT has recently garnered attention as it demonstrates the complexity of Eph/ephrin signaling and the context in which the cellular interactions take place. This will be discussed later, as well, but in short EphA2 is thought to regulate PI3K/AKT in breast cancer through p120RASGAP and inhibition of Ras [12].

Table 1. Known Eph receptor tyrosine kinases and their ligands.

Eph Receptors	Ephrin Ligands
A class	
EphA1	ephrinA1
EphA2	ephrinA2
EphA3	ephrinA3
EphA4	ephrinA4
EphA5	ephrinA5
EphA6	ephrinA6
EphA7	
EphA8	
EphA10	
B class	
EphB1	ephrinB1
EphB2	ephrinB2
EphB3	ephrinB3
EphB4	
EphB5	

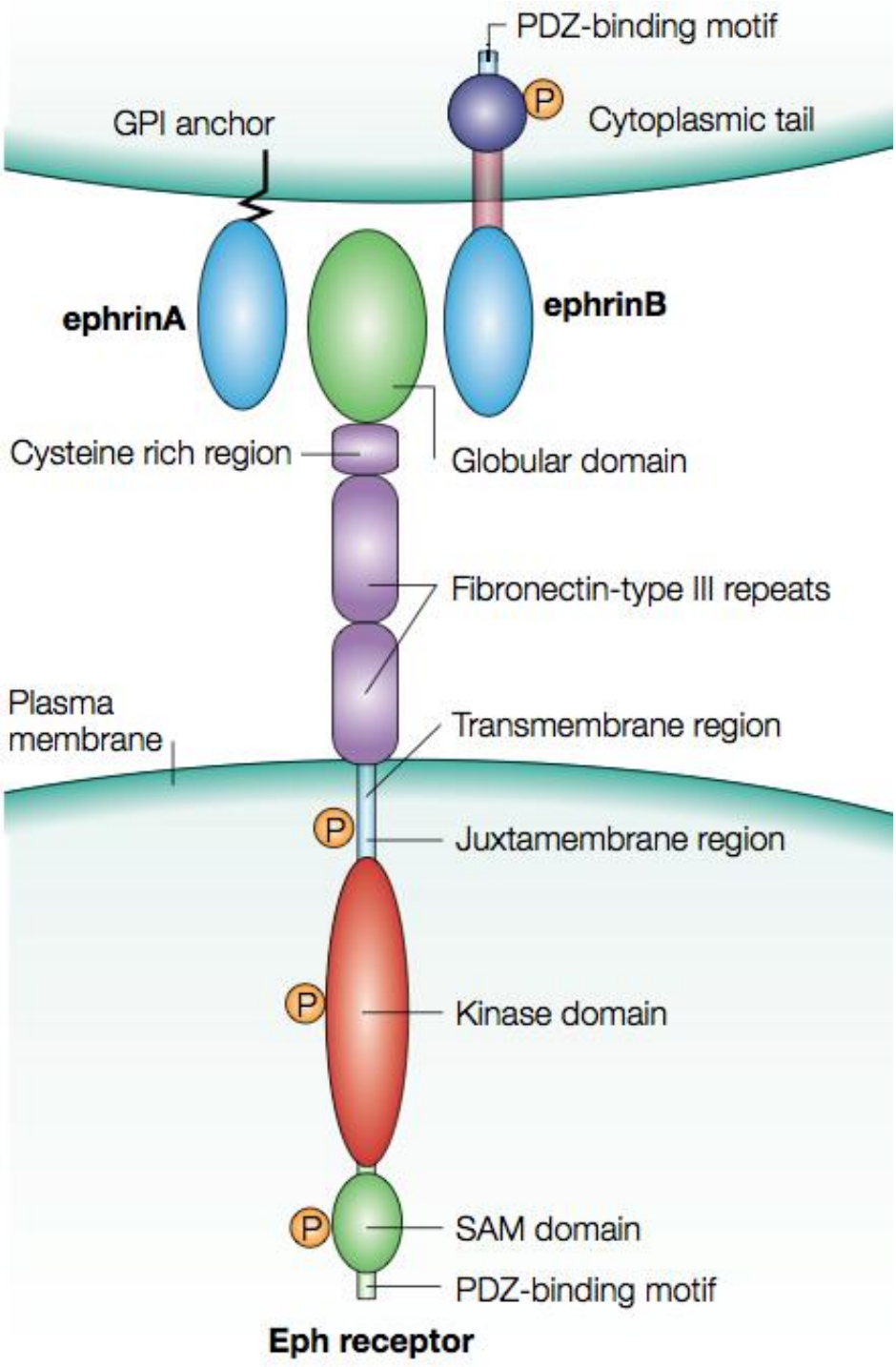


Figure 1. Eph receptor and ephrin ligands structure.
A diagram dictating major domains of the Eph receptor and ephrin ligands. The Eph receptor expressing cell is shown on the bottom in association with the ephrin-expressing cell on top. From [2].

The ability of Eph receptors to signal has continued to grow due to recently discovered interactions with other RTKs. Studies in our lab have demonstrated that EphA2 can physically interact with ErbB2, potentially increasing MAPK activation, while other labs have published reports of EphA2 and EGFR co-immunoprecipitation leading to increased cell motility [5, 13]. Likewise, a study of EphA4 suggested that EphA4 can phosphorylate EGFR via EphA4 peptide substrate studies [14]. Not all Eph receptor crosstalk with other RTKs involves the EGFR family or is confined to the A class of Eph receptors. Studies of EphB receptors revealed interaction with CXCR4 for AKT activation, as well as T cell recruitment through activation of the T cell receptor [15, 16]. Other examples of crosstalk are highlighted in Table 1. The importance of these crosstalk pathways is the different signaling pathways that can be facilitated based on interactions and which receptor is activated. The ability of other RTKs to be phosphorylated by Eph receptors or vice versa can have huge implications on therapies and the ability for resistance as recently described by Zhuang et. al. [17].

Bidirectional Signaling

The Eph family of RTK and ligands are unique in their requirement of cell-cell contact to create ligand-receptor interactions. They are further unique in their ability to signal through both the receptor expressing cell (forward signaling) as well as the ligand-expressing cell (reverse signaling) [1]. Eph signaling can control multiple functions in the cell: cell morphology, adhesion, proliferation,

migration, invasion, as well as more specialized functions like synaptic plasticity, immune function and bone remodeling (focus of Chapter 3) [1, 18]. Forward signaling proceeds like a traditional receptor tyrosine kinase propagating a signal following kinase activity via ligand binding. In contrast, reverse signaling occurs through the signal propagating through the ephrin ligand and ligand-expressing cell. EphrinB reverse signaling originates through phosphorylation of tyrosine residues in the cytoplasmic region of the ligand following receptor interactions. This signaling is dependent on SRC family kinases [32]. Studies show ephrinA ligands can also mediate their own signaling cascades likely through modulating integrin function and/or co-clustering with signaling molecules in specific membrane microdomains (e.g. clathrin-coated pits) [33, 34]. The mechanisms, however, of reverse signaling for the ephrinA class of ligands are less understood since they are without a cytoplasmic region and are GPI-linked to the cell membrane. Presumably, ephrinA-mediated reverse signaling requires the association of transmembrane signaling partners or internalization via a caveolin dependent mechanism.

Ligand Independent and Dependent Signaling

The complexity in Eph-ephrin signaling not only comes from bidirectional signaling but also from the ability of the receptor to signal independently of ligand binding. Many studies have been published showing classical receptor tyrosine

Table 2. Crosstalk Between Eph Receptors and Other Receptors

Eph Receptor	Crosstalk Receptor	Signaling Outcome	Reference
EphA	CXCR4 Receptor	Cdc42 Inhibition	[19]
EphA	Integrins	Rac1 inhibition	[20]
EphA2	EGF Receptors	Cell motility	[13]
EphA2	Claudin4	Claudin4 phosphorylation	[21]
EphA2	Integrins	FAK inhibition	[22]
EphA2	E-cadherin	EphA2 activation	[23]
EphA4	Integrins	Integrin activation	[24]
EphA4	FGF Receptor	MAPK activation	[25]
EphA4	EGF Receptor	EGF receptor phosphorylation	[14]
EphA8	Integrins	PI3K activation	[26]
EphB	NMDA Receptor (at synapses)	NMDA receptor phosphorylation	[27]
EphB	E-cadherin	E-cadherin	[28]
EphB2	Syndecan-2	Syndecan-2 phosphorylation	[29]
EphB2	L1	L1 phosphorylation	[30]
EphB2 and EphB4	CXCR4 Receptor	AKT activation	[16]
EphB2 and EphB3	Ryk Receptor (WNT signaling)	Tyrosine phosphorylation	[31]
EphB6	T Cell Receptor	T cell activation	[15]

kinase activation via ligand-dependent activity in signaling cascades. Most of these studies demonstrate the ligand as inhibitory to their respective signaling pathway when bound: Abl-Crk [35], Ras-Raf-MAPK [36], PI3K-Akt [37], integrin signaling [22] and small GTPase Rac activation [38]. Ligand independent signaling is demonstrated most often through crosstalk pathways involving Eph receptors. A recent report proposed EGFR activation of EphA2 as an effector for cell motility absent of ephrin ligand [13]. Our lab has recently demonstrated the ability of another EGFR family member ErbB2 to crosstalk with EphA2 leading to activation needed for breast tumorigenesis and progression [5] [17]. Another group has reported a direct interaction and response between FGFR and EphA4 as well as FGFR and EphA2 [25, 39].

Recent reports have demonstrated the ability of ligand-independent and ligand-dependent signaling to affect the same pathways in a manner that produces opposite outcomes. This aids in understanding why dramatically different responses are seen under different contexts of oncogenic signaling. Ten years ago, Bing Cheng Wang's group demonstrated that EphA2 activates focal adhesion kinase (FAK) in the absence of ephrinA1, while ephrinA1 dependent activation of EphA2 results in dephosphorylation of FAK and inactivation via the SHP-2 phosphatase [36]. The most recent description of ligand independent and dependent signaling within the same pathway involves a regulatory loop involving phosphorylation of serine 897 in EphA2 by Akt [37]. Activation of EphA2 with ephrinA1 suppresses Akt activation; limiting chemotactic

migration of glioma and prostate cancer cells, while EphA2 overexpression in the absence of ephrinA1 ligand promotes migration [37]. Thus, EphA2 is both an upstream negative regulator and a downstream activator of Akt depending on the presence or absence of ephrin ligand.

Ephs and Ephrins in Development

Eph RTKs and ephrins are expressed in almost all embryonic tissues and have been implicated in neuronal and vascular development [18, 40, 41]. They were first reported as axonal guidance cues in retinotectal topography, where Eph receptor and ligand expression helped develop gradients in the developing embryo that resulted in a repulsive behavior for retinal axons. This was demonstrated with temporal retinal axons expressing high EphA3 levels that would migrate and ultimately terminate in the anterior tectum where expression of its ligands, ephrinA2 and ephrinA5, was low [42, 43]. In vascular biology ephrinA1 transcripts were also detected in embryonic endothelial cells during embryonic development suggesting a role for the ligand in vasculature development [44-46]. EphrinA1 expression has also been detected in tumor vasculature as well as normal adult tissue vasculature by western blot (unpublished data). Further, a recently developed ephrinA1 KO animal demonstrated heart value development defects [47]. B class Eph receptors and ligands are also involved in cardiovascular development. EphB2, EphB3, EphB4, eprhrinB1 and ephrinB2 have all been implicated in the formation of the circulatory system in the mouse embryo [48-50].

Furthermore, the expression of Ephs and ephrins have been documented in important developmental stages involved in epithelial development. These developmental stages involve dispersal and rearrangement of the epithelia to promote gastrulation and segmentation. This is accomplished by spatial and temporal specific expression of Ephs and ephrins to form gradients that serve as boundaries in development through attractive and repulsive forces. For instance, EphA1 is expressed in varying combinations with ephrinA1 and ephrinA3 at different times in different regions during primitive streak formation where the germ layer will emerge [51]. Other epithelial based developmental features influenced by Ephs and ephrins include branched organs like the kidney, mammary gland, thymus and adult gut. In the thymus, EphA4 deletion results in abnormal organization of thymic epithelial cells resulting in severely affected T cell development [52]. In vitro studies of MDCK cells have revealed an important role for EphA2 and ephrinA1 in kidney branching [53], whereas the same group has observed the expression of several ephrin ligands in tissue extracts from embryonic kidneys [54]. Battle et. al. demonstrated Eph and ephrin signaling regulation of intestinal cell positioning of the epithelium in a very elegant study of the adult gut [55]. Furthermore, EphB4, ephrinB2, and EphA2 have all been shown as critical in mammary development. This is discussed in more detail below as well as serving as the focus of Chapter 2.

Mammary Gland Development

The mammary gland is a dynamic organ that undergoes drastic changes via growth and remodeling in response to hormonal cues at puberty and pregnancy.

The early mouse mammary gland epithelium develops after mid-gestation when milk lines develop from the forelimb to the hindlimb along which placodes form as precursor nipples (reviewed in [56]). Upon invagination by the placode into the mesenchyme, a rudimentary bud develops that later will support the mammary ductal tree. This rudimentary ductal tree (anlage) continues to grow with normal body growth until puberty commences, at which time hormonal signals (estrogen, progesterone, etc.) will cause a robust increase in proliferation. Upon hormonal stimulation, the epithelium responds by inducing proliferation at the tip of the epithelial duct in an area called the terminal end bud (TEB). The TEB consists of multiple cell types including cap cells, body cells and myoepithelial cells (Figure 2). Other specialized epithelial cells line the lumen and are referred to as luminal cells. These cells serve an integral part during lactation with the movement of milk through the ducts. The TEB, lumen and the milk producing alveolar cells are only one part of two distinct tissue compartments that constitute the mammary gland. The tissue surrounding the epithelial ducts is known as the stroma/connective tissue. This second compartment is composed of fibroblasts, adipocytes and other structural components that make up the mammary fat pad. Interactions between developing epithelial ducts and their adjacent mesenchymal stroma help regulate mammary gland morphogenesis through endocrine hormones and local paracrine interactions. Hormonal signaling induces proliferation at the distal tip of the TEB causing directional growth from the nipple while allowing bifurcation of the TEBs and secondary side-branches to sprout laterally from the trailing ducts. As the epithelial duct proliferates and moves

through the mammary fat pad, cells composing the cap differentiate to form the lining of the duct or undergo apoptosis to form the lumen of the duct. This process is continued until the entire fat pad is filled with a primitive ductal tree, upon which signals from the mesenchyme cause proliferation to cease. This leads to the TEB regressing into a quiescent terminal end duct (TED).

Proliferation of the epithelial cells occurs again during pregnancy in response to hormonal signaling (i.e. estrogen and progesterone). In order to meet the demand for milk production, the mammary epithelium undergoes dramatic expansion while simultaneously differentiating alveolar precursor cells into lobular alveoli that are capable of secreting milk for lactation. The epithelium (including lobular alveoli) completely fills the mammary gland during lactation and will remain that way until the final stage of offspring development (Figure 3). Involution occurs when lactation ceases after the offspring are weaned and is marked by massive cell death (apoptosis) and remodeling of the alveolar compartment back to a quiescent state. The return to this quiescent ductal state keeps the epithelium intact for subsequent rounds of pregnancy and lactation (reviewed in [57, 58]).

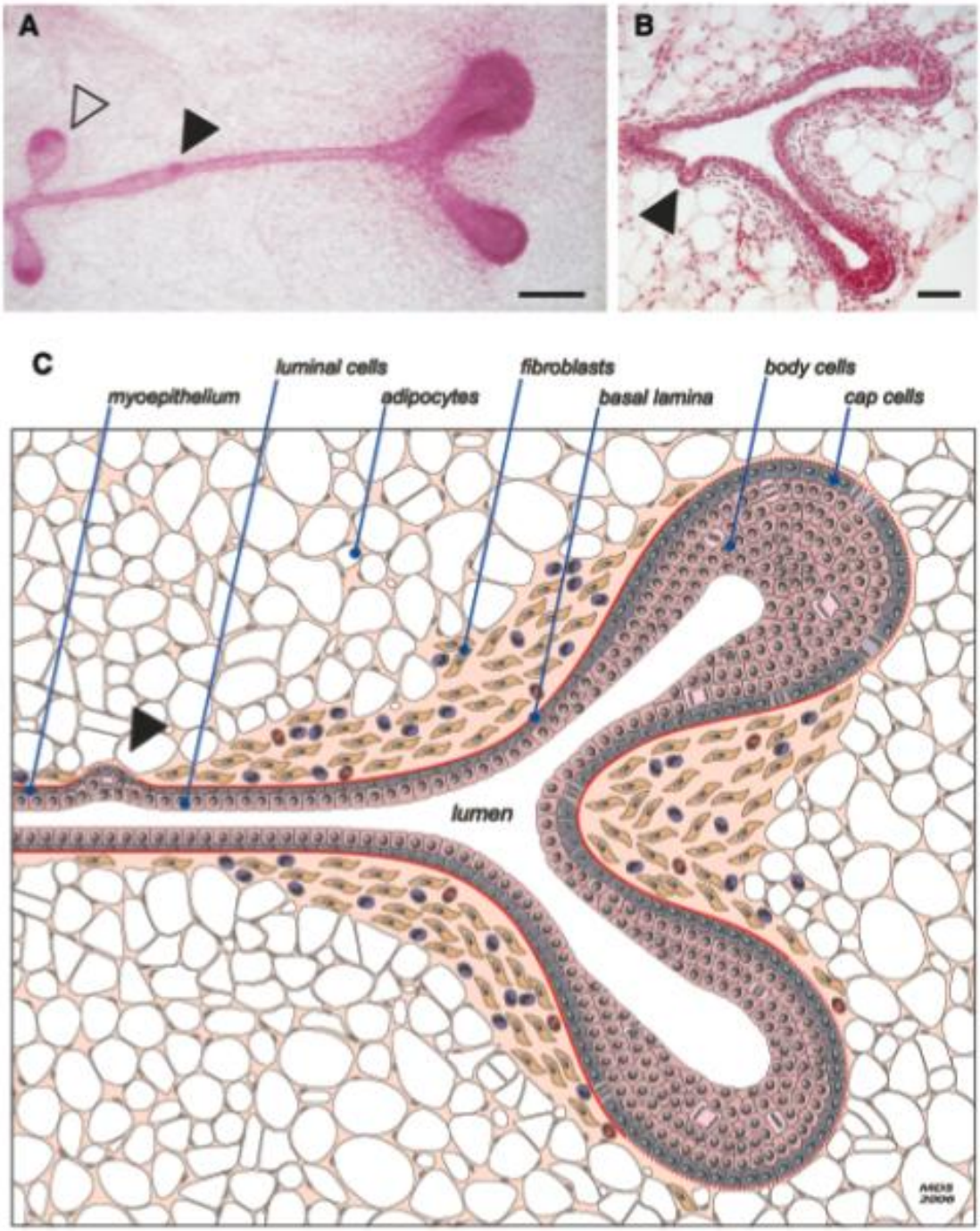

A
B
C
myoepithelium
luminal cells
adipocytes
fibroblasts
basal lamina
body cells
cap cells
lumen

Figure 2. Mammary gland structure and morphology. (A) High magnification carmine alum-stained whole mount of TEB that has recently bifurcated to form two new primary ducts. Two secondary side-branches are also present along the trailing duct (open arrowhead), as is an area of increased cellularity that may represent a nascent lateral bud (closed arrowhead). Increased stromal cellularity is apparent around the bifurcating TEB. Scale bar 200mm. (B). Hematoxylin and eosin-stained section of bifurcating TEB with an early lateral side-branch (closed arrowhead). Scale bar 100mm (Image courtesy of A.J. Ewald UCSF). (C) Schematic diagram depicting the major features of a bifurcating TEB. Notable features include the considerable proliferative activity (mitoses) within the TEB, the single layer of TEB cap cells and multilayered pre-luminal body cells, the characteristic presence of a fibroblast-and collage-rich stromal collar surrounding the neck of the bifurcating TEB, and its conspicuous absence beyond the invading distal cap of each new TEB. An increased number of macrophages and eosinophils is also shown. Although there is no evidence that normal ductal cells ever cross the basal lamina, thinning of the basement membrane (dashed lines) at the leading edge of the invading duct may reflect partial enzymatic degradation and/or incomplete de novo synthesis of the basal lamina. (From [57])

The ability of the mammary gland to replenish cells through cycles of pregnancy, lactation and involution has been attributed to stem cells found in the mammary gland ([59], reviewed by [60]). Studies have shown epithelial cells are lost during development as they differentiate into alveolar or ductal systems. They are also lost due to shedding into the milk during lactation. Mammary gland integrity must be maintained through replacement of these cells. Studies have revealed the existence of self-renewing multipotent mammary stem cells and transplantation studies of epithelium fragments in mice and rats, upon removal of endogenous epithelial components, have clearly demonstrated their renewing capabilities [60-63].

Eph Receptors in Mammary Gland Development

Mammary epithelial morphogenesis is a complex developmental process during which extensive networks of branched ducts form from a rudimentary epithelial bud. This process is termed branching morphogenesis and is regulated by endocrine hormones and local paracrine interaction between the developing epithelial ducts and their adjacent mesenchymal stroma. Expression of multiple Eph family receptors and their ligands has been reported in the mammary gland. Ephrin-B2 is expressed on the luminal cells, and its receptor, EphB4, is expressed complementarily on myoepithelial cells in mice. The expression of EphB4 and ephrinB2 is dependent on estrogen and is regulated during the estrus cycle [65]. Forced overexpression of EphB4 under the control of the mouse.

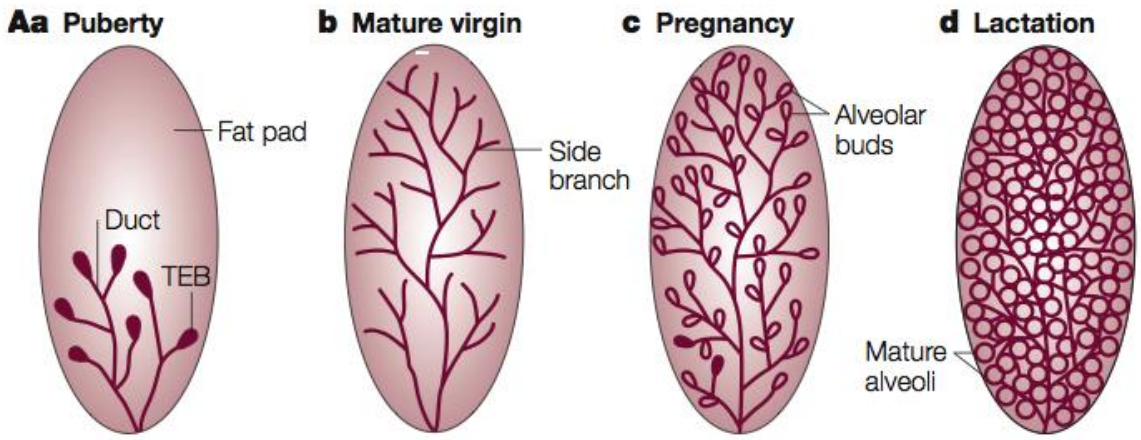


Figure 3. Mammary gland development during puberty, pregnancy and lactation. Schematic (a-d) presentation of the different stages of mammary gland development. A rudimentary ductal design within the mammary fat pad is visible at birth, which grows at the same rate as the animal until the onset of puberty. (a) During puberty, cyclical hormone production accelerates ductal outgrowth causing club-shaped structures (TEB) where the highest levels of cell division occur to appear at ductal tips. (b) In mature virgin, the entire fat pad is filled with a regularly spaced system of primary and secondary ducts, with side branches that form and disappear in each oestrous cycle. (c) Hormonal changes that occur when pregnancy begins increase cell proliferation and the formation of alveolar buds. (d) During lactation, alveoli are fully matured and the luminal cells synthesis and secrete milk components in the lumina. Following lactation the mammary gland will undergo massive apoptosis during involution to revert back to a mature virgin gland structure. (From [64]).

mammary tumor virus (MMTV) promoter/enhancer induced a delayed development of the mammary epithelium at puberty and during pregnancy, with untimely epithelial apoptotic cell death during pregnancy and abnormal epithelial DNA synthesis at early post-lactation involution, indicating a disturbed response to proliferative/ apoptotic signals [66].

In addition to EphB4, developmentally controlled expression of EphA2 in the mammary epithelium has also been reported [67, 68]. Loss of EphA2 receptor resulted in decreased penetration of mammary epithelium into the fat pad and reduced epithelial proliferation and inhibition of epithelial branching, suggesting a positive role for EphA2 during normal mammary gland development [69]. EphA2 is also expressed in human mammary epithelial cells [10, 70-72]. Fournier et al. analyzed gene expression in two non-malignant human mammary epithelial cell lines in 3D cultures. When these cells underwent growth arrest and differentiated into polarized acini, EphA2 levels were significantly decreased [73]. This is consistent with the observation that EphA2 is expressed at low levels in normal mammary gland epithelium, whereas expression increases in breast cancer [74]. Indeed, analysis of a set of 19 genes that were down regulated in differentiated acini of human mammary epithelial cells in 3D cultures against 2 independent breast cancer microarray datasets revealed that increased EphA2 levels are associated with poor patient prognosis [72, 73]. Taken together, these data suggest that EphA2 is required for mammary gland morphogenesis and increased EphA2 expression in human breast cancer is associated with tumor

cell malignancy and poor patient survival.

Dysregulation of Ephs and Ephrins in Cancer

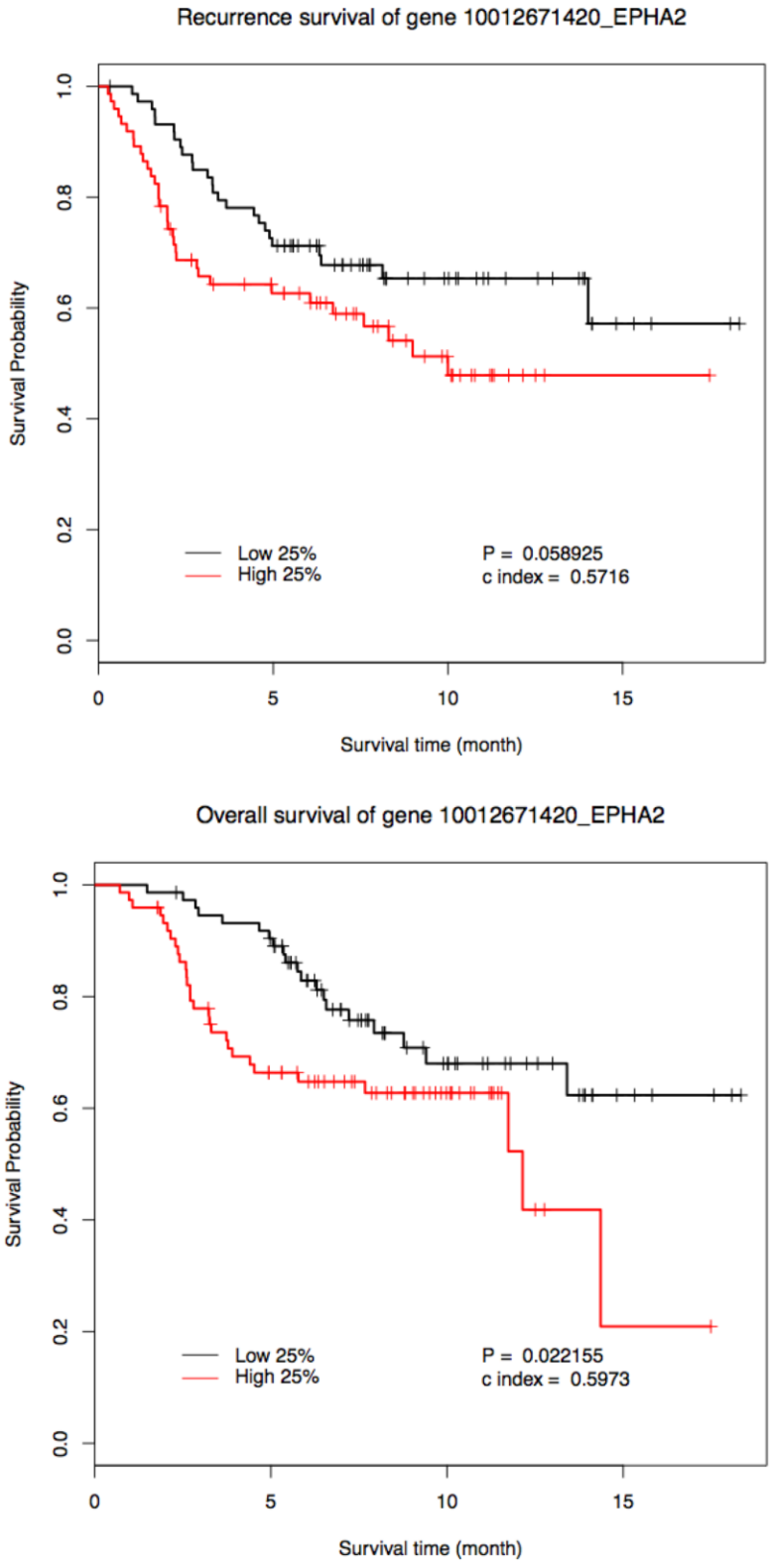
As previously stated, multiple Ephs and ephrins have critical roles in prenatal and postnatal development. Due to the fact that many cancer studies have revealed that aberrant developmental signaling pathways often contribute to tumorigenesis, a strong push was made looking into the potential for dysregulation of Eph and ephrin signaling in cancer. Eph receptors are often overexpressed in many human cancers, including melanomas, sarcomas, leukemias, brain tumors, and breast cancer [75]. Current data demonstrate that Eph receptors and ephrins function in both tumor cells and the tumor microenvironment, with dual roles in tumor suppression and tumor promotion. These observations suggest the feasibility in screening for Eph receptors and/or ligands as predictors of prognosis in patients. EphA2, for example, has been linked to increased malignancy and poor clinical prognosis in breast cancer [72], non-small cell lung cancer [76], esophageal squamous cell carcinoma [77], cervical squamous cell carcinoma [78], renal cell carcinoma [79], glioblastoma multiforme [80], and endometrial cancer [81]. Overexpression of EphA4 in gastric cancer is associated with shorter survival [82] and increased expression of EphA7 is associated with adverse outcome in primary and recurrent glioblastoma multiforme [83]. Poor prognosis and/or reduced survival is not only associated with the A class of receptors. Reports on EphB4 overexpression suggest poor overall survival in patients with ovarian cancer and head and neck

squamous cell carcinoma [84, 85]. Using the van de Vijver dataset we were able to confirm that elevated levels of EphA2 were associated with increasing malignancy and poor prognosis in breast cancer, as well as being mutually exclusive for EphA2/ephrinA1 expression in metastatic cancer (Figure 4, unpublished data).

Role of Eph Receptor in Breast Cancer Promotion

Results from high throughput screens revealed many Eph receptors were overexpressed in multiple types of human tumors [1, 74]. However, in breast cancer, the number of Eph receptors found to be expressed were limited to EphA2 and EphB4, the two Eph receptors most extensively studied with highest degree of aberration [86]. EphA2 is expressed at low levels in normal human breast epithelium [67, 87] and overexpressed in 60-80% of breast cancers [70, 71, 88](Brantley-Sieders and Chen, unpublished data). Experimentally induced overexpression of EphA2 resulted in malignant transformation of non-transformed MCF10A breast cells and enhanced malignancy of pancreatic carcinoma cells [71]. Conversely, siRNA-mediated inhibition of EphA2 expression impaired malignant progression of pancreatic, ovarian and mesothelioma tumor cell lines, and overexpression of dominant-negative EphA2 constructs suppressed growth and metastasis of 4T1 metastatic mouse

Figure 4. Kaplan-Meier analysis for EphA2 in breast cancer. Recurrence survival and overall survival was defined as death due to any cause. (log-rank test) from Van de Vijver et. al. [93]



mammary adenocarcinoma cells *in vivo* [11, 89-91]. To determine whether EphA2 plays a causative role in breast cancer initiation and metastatic progression, EphA2 knockout mice were crossed to MMTV-Neu transgenic animals that express a rat homologue of the ErbB2 receptor tyrosine kinase. Loss of EphA2 impairs both tumor initiation and lung metastasis in MMTV-Neu mice [5]. Similarly, EphB4 levels are also elevated in human breast cancer [87]. EphB4 knockdown inhibited breast cancer survival, migration, and invasion *in vitro* and tumor growth in a xenograft model *in vivo* [92]. Furthermore, overexpression of EphB4 in the mammary epithelium accelerates tumor onset and lung metastasis in MMTV-Neu animals [66]. Taken together, these studies indicate a role for Eph receptor in tumor promotion.

Although previous studies indicate a role for Eph receptors in tumor promotion, the mechanisms regulating this oncogenic function are not entirely clear. In many instances, Eph receptors in tumor cells are underphosphorylated in spite of overexpression. This could be due to increased activity of phosphotyrosine phosphatases or loss of E-cadherin in tumor cells. As E-cadherin regulates cell surface localization of EphA2 and/or loss of cell-cell contacts prevent interaction with endogenous ephrin ligands, which often results in internalization/degradation of receptors after activation by ligand [23, 94, 95]. As both Eph receptors and ligands are membrane anchored and reside in separate microdomains on the cell surface, loss of cell-cell adhesion in tumor cells impairs activation of Eph receptors by ephrins on adjacent cells [96, 97]. Thus, the oncogenic activity of Eph receptors appears to be ligand independent.

High levels of EphA2 receptor have been shown to physically and functionally interact with the EGF receptor and ErbB2, promoting Erk and RhoA GTPase activity [13]. These data suggest that crosstalk between Eph receptors and other oncogenic pathways promotes tumor cell malignancy in an ephrin-independent manner. Furthermore, a high level of EphA2 was found to upregulate matrix metalloproteinase 2 (MMP2) [98] and extracellular matrix protein fibronectin [99]. Modulation of tumor cell interaction with the microenvironment may also contribute to Eph receptor function in tumor promotion.

Role of Eph Receptors in Tumor Suppression

Many studies demonstrate a role of Eph receptors in tumor suppression. Stimulation of EphA receptors with soluble ephrinA1-Fc ligand reduced Erk phosphorylation in tumor cell lines, fibroblasts, and primary aortic endothelial cells and suppressed growth of primary keratinocytes and prostate carcinoma cells [8, 36]. Macrae et al. also reported that treatment of human breast cancer cell lines with ephrinA1-Fc attenuated EGF-mediated phosphorylation of Erk and inhibited transformation of NIH3T3 cells expressing v-erbB2 [8]. In addition, EphA2-deficient gene-trap mice displayed increased susceptibility to chemical carcinogen-induced skin cancer, accompanied by increased tumor cell proliferation and phosphorylation of Erk [100]. These data suggest that ephrin-A-induced EphA2 receptor forward signaling inhibits tumor malignancy.

In addition to EphA2, EphB4 forward signaling also appears to inhibit tumor progression. Systemic delivery of ephrinB2-Fc inhibits the growth of MDA-MB-

435 tumor xenografts [35]. EphB4 forward signaling activates the Abl/Crk pathway to inhibit tumor cell growth and motility in breast cancer cells [35]. Furthermore, EphB receptor signaling is also able to suppress tumor expansion in colon cancer. Overexpression of a dominant negative EphB2 cytoplasmic truncation mutant or knockout of EphB3 or ephrinB1 in the intestinal epithelium significantly increases tumor numbers and tumor invasiveness in the $APC^{min/+}$ model [28, 101]. EphB receptors have been proposed to compartmentalize the expansion of colon cancer cells through a mechanism dependent on E-cadherin–mediated adhesion [28].

In summary, ephrin-induced Eph receptor forward signaling in non-transformed mammary epithelial cells appears to transduce an inhibitory signal that may keep cells quiescent and non-invasive [8, 35, 36]. Upon tumor initiation, Eph receptor expression is up-regulated by oncogenic signaling pathways such as the Ras-MAPK pathway in breast cancer or the Wnt-ß-catenin pathway in colon cancer, whereas their ephrin ligands are often downregulated [8, 55] or unable to bind to receptor due to loss of cell-cell adhesion [23]. Crosstalk between elevated Eph receptors and other oncogenes, such as the ErbB family of receptor tyrosine kinases leads to enhanced cell proliferation and tumorigenesis, presumably independently of ephrin stimulation [13].

Eph Receptors and Ephrins in Tumor Angiogenesis

Tumor angiogenesis is critical for growth, survival, and malignant progression of tumors. Tumor vessels not only supply the nutrients and oxygen necessary for

tumor cell growth and survival, but also actively promote malignant progression by providing an entry point into the circulation for the dissemination of metastatic cells [102]. In addition to regulating developmental angiogenesis, Eph receptors and ephrins have also emerged as critical regulators of tumor angiogenesis. The first ligand discovered for the Eph receptors, ephrinA1, is a tumor necrosis factor alpha (TNF-α) inducible gene in endothelial cells [103]. Early studies demonstrated that ephrinA1 promotes angiogenic responses *in vitro* and corneal neovascularization *in vivo*. EphrinA1 is expressed in developing embryonic and tumor vasculature [45, 70, 87]. More importantly, ephrinA1 is further induced by hypoxia in tumors that are resistant to anti-VEGF therapy [104]. Interestingly, in recently generated ephrinA1 KO animals, mice deficient for the A1 ligand survive to adulthood with only minor heart valve defects [47], suggesting that other ephrinA ligands can functionally compensate for the loss of ephrinA1 in vascular development. It remains to be determined whether tumor angiogenesis is affected in these mice.

EphA2, a major receptor for ephrinA1 in vascular endothelial cells, plays a significant role in promoting tumor angiogenesis. Implantation of tumor cells subcutaneously or into the mammary gland of EphA2-deficient host mice results in reduced tumor volume, microvascular density, and lung metastasis [105]. These results suggest that loss of EphA2 in the tumor microenvironment impairs tumor angiogenesis and metastatic progression [105, 106]. Indeed, EphA2-deficient vascular endothelial cells fail to migrate and assemble in response to angiogenic cues *in vitro* and are unable to incorporate into tumor blood vessels

when they are co-transplanted with tumor cells *in vivo* [106, 107], indicating a critical function for EphA2 in tumor angiogenesis. In contrast to the complex effects of Eph signaling in tumor cells, ephrin-Eph bi-directional signaling in vascular endothelial cells promotes tumor angiogenesis. Brantley-Sieders et al. showed that EphA2 receptor forward signaling regulates endothelial cell migration and assembly through PI3 kinase-mediated Rac1 GTPase activation [106]. A yeast two-hybrid screen for EphA2 interacting proteins revealed that Vav2 and Vav3 guanine nucleotide exchange factors are recruited to activated EphA2 receptor and subsequently elevate Rac1-GTP levels [48]. Loss of Vav2 and Vav3 inhibits Rac1 activity and ephrinA1-induced angiogenic responses both *in vitro* and *in vivo* [48]. Furthermore, Fang et al. mapped phosphorylated tyrosine residues of EphA2 in vascular endothelial cells [3]. EphrinA1-induced phosphorylation of Y587 and Y593 in the EphA2 receptor recruits Vav2 and Vav3 exchange factors, whereas phosphorylation of Y734 provides a docking site for the p85 regulatory subunit of PI3 kinase [107]. EphA2-null endothelial cells reconstituted with EphA2 mutants lacking these binding sites fail to activate Rac1 GTPase, are defective in cell migration and assembly *in vitro* and are unable to incorporate into tumor vasculature *in vivo*. These results suggest a critical role for these tyrosine phosphorylation sites in transducing EphA2 forward signaling in vascular endothelial cells and validate the involvement of PI3 kinase-dependent activation of Vav exchange factors and Rac1 GTPase in ephrinA1-induced angiogenesis.

Gene targeting studies have established ephrinB2 and EphB4 as key regulators of embryonic vascular development [49, 108]. EphrinB2 expression has also been observed in tumor vasculature in a variety of tumor types, suggesting that this ligand may regulate tumor neovascularization [108-110]. In support of this hypothesis, A375 melanomas form smaller, less vascularized tumors in the presence of the soluble, monomeric EphB4 extracellular domain *in vivo* [111]. Soluble EphB4 may act, at least in part, by preventing binding of tumor cell EphB receptors to ephrinB2-positive endothelium, thus disrupting tumor angiogenesis. Further support for this hypothesis is provided from studies in which overexpression of a truncated cytoplasmic deletion EphB4 receptor construct produced increased tumor growth and vascularity in mammary tumors, likely through ephrinB2 mediated reverse signaling in host endothelium [110]. Upregulation of ephrinB1 expression has been reported in hepatocellular carcinoma, and overexpression of ephrinB1 enhances tumor neovascularization *in vivo* [112]. Although proliferation of ephrinB1 overexpressing cells was not affected in culture, soluble ephrinB1-Fc enhanced endothelial cell proliferation and migration *in vitro*, suggesting that at least one function of ephrinB1 in tumor progression involves facilitation of tumor angiogenesis [112, 113]. Taken together, these studies reveal a critical role for B class receptors and ligands in tumor progression and vascular recruitment for multiple types of human cancer.

Breast Cancer Metastasis

Although the overall five-year survival rate has increased due to early detection and advances in treatment, women diagnosed with more advanced and/or aggressive forms of breast cancer have only about a quarter chance of reaching the five-year survival mark. This low survival rate is usually not due to the primary tumor, but is often a result of cancer cells disseminating to distant organs [114, 115]. These disseminating cancer cells from primary breast tumors often colonize the same sites: lung, liver, brain, and bone [116]. This characteristic ability of breast cancer cells to metastasize to certain organs but not others was first observed by Stephen Paget who argued distribution compatibilities between disseminated tumor cells (the seed) and certain organs (the soil) could not be merely by chance [117]. Despite the more than a century of research following Paget's metastasis theory, our knowledge of the molecular mechanisms underlying breast cancer metastasis to specific organ sites such as bone remains limited.

The skeleton is a favored site of metastasis for many tumors. Studies show approximately 70% of patients that die from breast or prostate cancer have bone metastasis [118]. Metastasis to bone is often undetected in early stages of breast cancer but at later stages it can invariably lead to bone pain, nerve compression, and bone fractures, with extensive bone destruction leading to hypercalcemia that increases the mortality and morbidity of patients [118]. Despite our current understanding of bone development and the aberrations associated with breast cancer, only palliative treatment options are available to the patient once metastases are discovered in the bone, as there is no cure.

Physiology of the Bone: Bone Components

Bone is a specialized type of connective tissue that provides structural support, protective functions, and regulation/reservoir of calcium and growth factors in the body [119]. Two types of bone are present within the skeleton, cortical and trabecular. Both types contain the same composition, mostly type I collagen with a few non-collagenious proteins like osteopontin (OPN) and osteocalin, though they are organized differently to provide for different functions within the skeleton [119]. Cortical bone is dense and tightly compacted to serve as a protective layer around the bone and while also serving to support the weight load of the body. In contrast, trabecular bone is loosely organized and porous. It is located in the interior of the bone near the ends, and is metabolically active and will undergo a higher turnover rate than cortical bone [119]. Long bones in the body are divided into three portions of the diaphysis, metaphysis, and epiphysis. The ends of the bone, ephiphysis, are located above the growth plate and are the portion of the bone that elongates during growth. The diaphysis is the long narrow portion of the bone composed mainly of cortical bone, in contrast to the metaphysis, located just below the growth plate, composed predominantly of trabecular bone (Figure 5).

Bone Resorption

Bone remodeling takes place throughout life and is necessary for structural maintenance and skeletal repair as well as for calcium homeostasis.

The process of remodeling consists of coupled processes of bone resorption and formation, discussed in detail later. The sequence of events mediating remodeling occurs synchronously throughout the skeleton and the resorption of "old" bone to be replaced with "new" bone occurs in the same place at the same time so there are no changes to the shape of the bone. The importance of local control on this process through cytokine and hormonal regulation can be demonstrated through genetically engineered animal models designed to under- or over-express cytokines, hormones and/or their receptor [120].

Constant remodeling of bone is facilitated by three cell types: osteoblast, osteoclasts and osteocytes. Together, these cells tightly regulate bone resorption and deposition. Osteoblasts are derived from mesenchymal stem cells and synthesize new bone by providing for the new bone matrix and aiding in mineralization of this new matrix. Osteoblast precursors (MSC's) can be induced to proliferate and differentiate into mature osteoblasts through cell-cell interactions, cell-extracellular matrix contacts, and many soluble factors such as chemokines, cytokines, hormones and growth factors [121]. Transcription factors RUNX2 and OSTERIX, bone morphogenic proteins (BMPs), and local growth factors (IL-1, TNFα, PTH) all stimulate differentiation of osteoblasts and bone formation [122-124]. After synthesis of new bone, mature osteoblasts either undergo apoptosis or become embedded in the bone as osteocytes. During bone deposition, osteoblasts secrete multiple factors (BMPs, IGF, TGF-β, and FGF) that become incorporated into the bone thus making bone a rich reservoir of growth factors.

Osteocytes, which are derived from osteoblast embedded in the bone matrix, determine the location of osteoclasts in the bone. It is believed that osteocytes can sense microcracks and microfractures in the bone as well as mechanical load deficiencies that trigger osteoclasts differentiation and recruitment (reviewed in [125, 126]).

Osteoclasts are cells responsible for bone degradation (osteolysis) and are derived from hematopoietic stem cells, which also give rise to monocytes, dendritic cells and macrophages [127, 128]. Osteoclasts form from precursor cells fusing together in response to proliferation (often induced by M-CSF) resulting in large multinucleated cells [129] (Figure 6). Osteoclasts become functional and are activated by RANKL expressed on osteoblasts or released by osteoblasts. These activated osteoclasts bind to the bone matrix through integrin binding by $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_2\beta_1$ [127, 128]. The sealed compartment formed between the bone surface and the osteoclast is created by reorganization of the actin cytoskeleton in the osteoclast [127, 128]. In this sealing zone an acidic

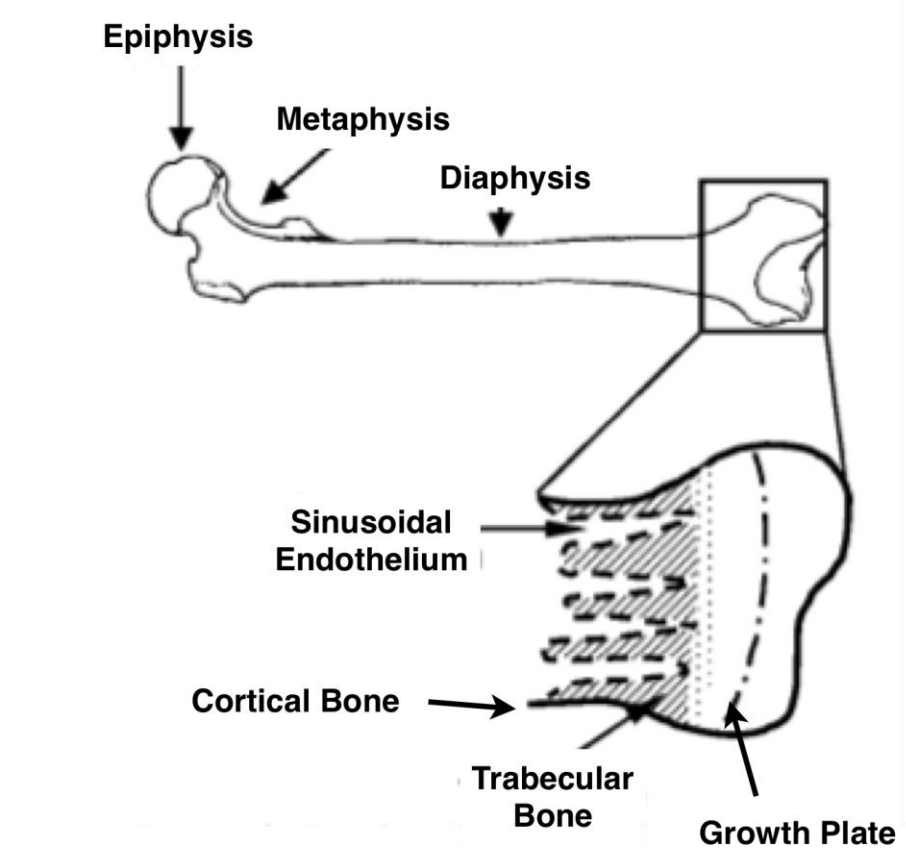


Figure 5. Long Bone Anatomy. Diagram of a long bone indicating the major regions and the major structures within the metaphysis, i.e. trabecular bone, cortical bone, growth plate and the sinusoidal endothelium. Adapted from [123]

environment is achieved by the action of a v-type H^+-ATPase electrogenic proton pump and Cl^- channel [127, 128]. Proteolytic enzymes, like cathespin K, degrade the bone matrix, mostly type I collagen, which is exposed as a result of acidification [130].

Bone Remodeling

Bone remodeling is a critical, tightly regulated process responsible for replacing damaged or old bone with new bone [131]. The coupling of bone resorption with bone formation during remodeling helps in achieving a balance between the two as different cells are responsible for different functions. Dysregulation of this coupling can lead to many pathological conditions like osteoporosis, osteopetrosis, and rheumatoid arthritis. In order for coupling to occur osteoclast and osteoblasts must communicate (reviewed in [132]). There are three models for communication between osteoclasts and osteoblasts. 1. Osteoclasts and osteoblasts can make direct contact allowing membrane-bound receptors and ligands to interact and induce activation of intracellular pathways. 2. Osteoclasts and osteoblasts can form gap junctions that allow for the diffusion of small water-soluble molecules between the cells. Signaling via paracrine activity of growth factors, cytokines, and chemokines can also occur this way. 3. Liberated growth factors from osteoclast resorption of bone can lead to communication between the cells in form of positive and negative feedback loops [133].

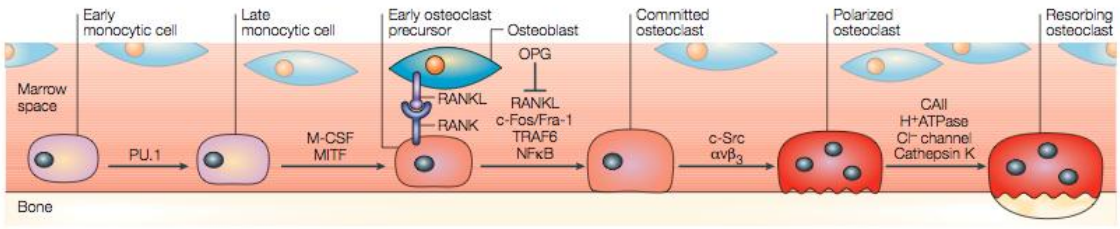


Figure 6. The osteoclast is a member of the monocyte/macrophage family. Early nonspecific differentiation along the osteoclast pathway is dependent on PU.1 and the MITF family of transcription factors, as well as the macrophage proliferation and survival cytokine M-CSF. Activation of RANK by osteoblast-expressed RANK ligand (RANKL) commits the cell to the osteoclast fate, which is mediated by signaling molecules such as AP-1 transcription factors, tumor necrosis factor receptor associated factor 6 (TRAF6), nuclear factor κB (NFκB), c-Fos and Fra-1. RANKL-stimulated osteoclastogenesis is inhibited by the RANKL decoy receptor osteoprotegerin (OPG). The initial event in development of the resorptive capacity of the mature osteoclast is its polarization, which requires c-Src and the αvβ3 integrin. Once polarized, the osteoclast mobilizes the mineralized component of bone. Bone mobilization is achieved through the acidifying molecules, carbonic anhydrase II (CAII), an electrogenic H^+ATPase and a charge-coupled Cl^- channel. Cathepsin K mediates bone organic matrix degradation. (From [130])

Soluble factors like PTH, PTHrP, TNFα, Il-1, IL-11, PGE2, and 1,25-$(OH)_2$ vitamin D3 can enhance osteoclastogenesis through RANKL-mediated induction of osteoblasts [132]. Using genetically manipulated mice and soluble RANKL for rescue, it is well established that RANK signaling is critical for osteoclastogenesis [134, 135]. The same osteoblasts that can induce osteoclast differentiation via RANKL can inhibit osteoclastogenesis by release of a decoy receptor for RANKL, OPG [136]. The ratio of OPG:RANKL is indicative of osteoclastogenesis activity with more OPG interfering with osteoclasts-osteoblasts interactions to inhibit differentiation and fusion of osteoclast precursors.

The "Vicious Cycle" of Bone Metastasis

In order for a primary breast tumor to metastasize to a distant organ i.e. bone, tumor cells from the primary tumor must go through a series of coordinated steps. Breast cancer cells must invade the surrounding host stromal tissue and break away from the primary tumor, intravastate into the blood stream, survive in the blood stream, adhere to the blood vessel wall, extravastate from the blood circulation, and colonize this secondary (metastatic) site (Figure 7). Multiple genes regulate the ability of cells to undergo metastasis and this is reviewed nicely in [116].

Once breast tumor cells have established themselves in the bone microenvironment they encounter a permissive environment for growth via the richness of growth factors, cytokines, and chemokines that are stored or present in the bone. These factors induce tumor cell growth that allows for the release

of PTHrP, IL-1, -6, -8, and -11 that activates osteoblast to produce RANKL. RANKL secreted from osteoblasts binds to the RANK receptors on osteoclast inducing

differentiation, activation, and resorption of the bone. Growth factors like TGF-β and IGF plus minerals like calcium (Ca^{2+}) are released from bone when it is degraded, which stimulates the growth of cancer cells with subsequent release of more PTHrP and interleukins thus establishing a positive feedback loop (Figure 8). Therefore, osteolytic lesions ultimately result from sustained osteoclast activation uncoupled from bone restoring osteoblast function [137].

Eph Receptors in Bone Biology

A role for ephrin ligands and Eph receptors in bone biology was recently discovered. The first example of Eph receptors and ephrin ligands in bone biology was established in the B class with the studies presented by Compagni et al. Using an ephrinB1 knockout mouse model the investigators were able to see skeletal abnormalities including cleft palate, skull shortening, asymmetric pairing of the ribs, and sternebral fusions [138]. Other studies of ephrinB1 knockout mice by an independent group revealed similar skeletal abnormalities including limb bud and digit malformations [139]. Furthermore, ephrinB1 mutant mice with targeted PDZ biding mutations revealed the ephrinB1 ligand was targeted to cells in the mesenchymal lineage and cells expressing the ephrinB1 mutation had decreased bone mass and size [140]. Human studies have found ephrinB1 genetic mutations are associated with craniofrontonasal syndrome [141, 142].

Recent studies have demonstrated that ephrinB2 expressed on osteoclasts is inhibitory for osteoclast differentiation in contrast to EphB4 on osteoblasts being stimulating [143]. A class Eph receptors have also recently been implicated in bone homeostasis. A recent report has demonstrated the role of EphA4 as a guidance cue for osteogenic precursor cells during calvarial bone growth through Twist1 [144]. Irie et. al. have shown the ephrinA2-EphA2 interaction between osteoclast precursors and osteoblasts enhances osteoclastogenesis while inhibiting osteoblast differentiation [145].

Eph Receptors in Cancer-Induced Bone Disease

The increasing evidence of Eph ligand and receptor activities in bone biology raises the possibility of these Eph and ephrin interactions having a role in aberrant bone remodeling. Breast cancer and multiple myeloma are associated with bone metastases that exhibit high levels of osteolysis while prostate cancers usually have higher levels of bone formation. In multiple myeloma, osteolysis is driven by lack of EphB4 expression on osteoblasts causing an inhibition of new bone. A class Eph receptors and ephrin ligands are also implicated in cancer-induced bone dysregulation. A tissue microarray of prostate cancer metastasis foci in lymph node, liver, and bone revealed a decrease expression level of

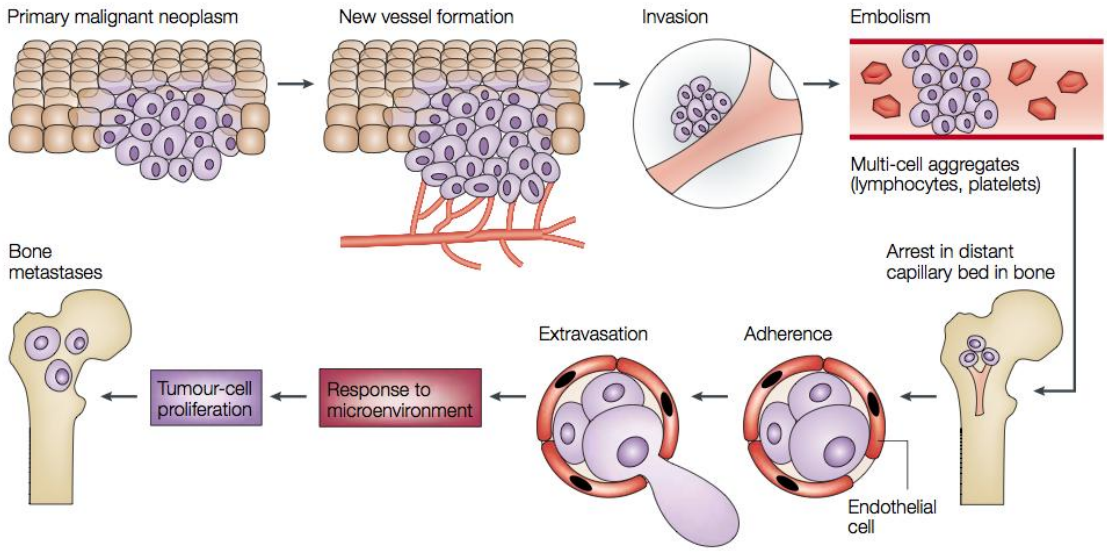


Figure 7. The steps involved in tumor-cell metastasis from a primary site to the skeleton. The primary malignant neoplasm promotes new blood vessel formation, and these blood vessels carry the cancer cells to capillary beds in bone. Aggregates of tumor cells and other bloods cells eventually form embolisms that arrest in distant capillaris in bone. These cancer cells can then adhere to the vascular endothelial cells to escape the blood vessels. As they enter the bone, they are exposed to factors of the microenvironment that support growth of metastases. (From [114]).

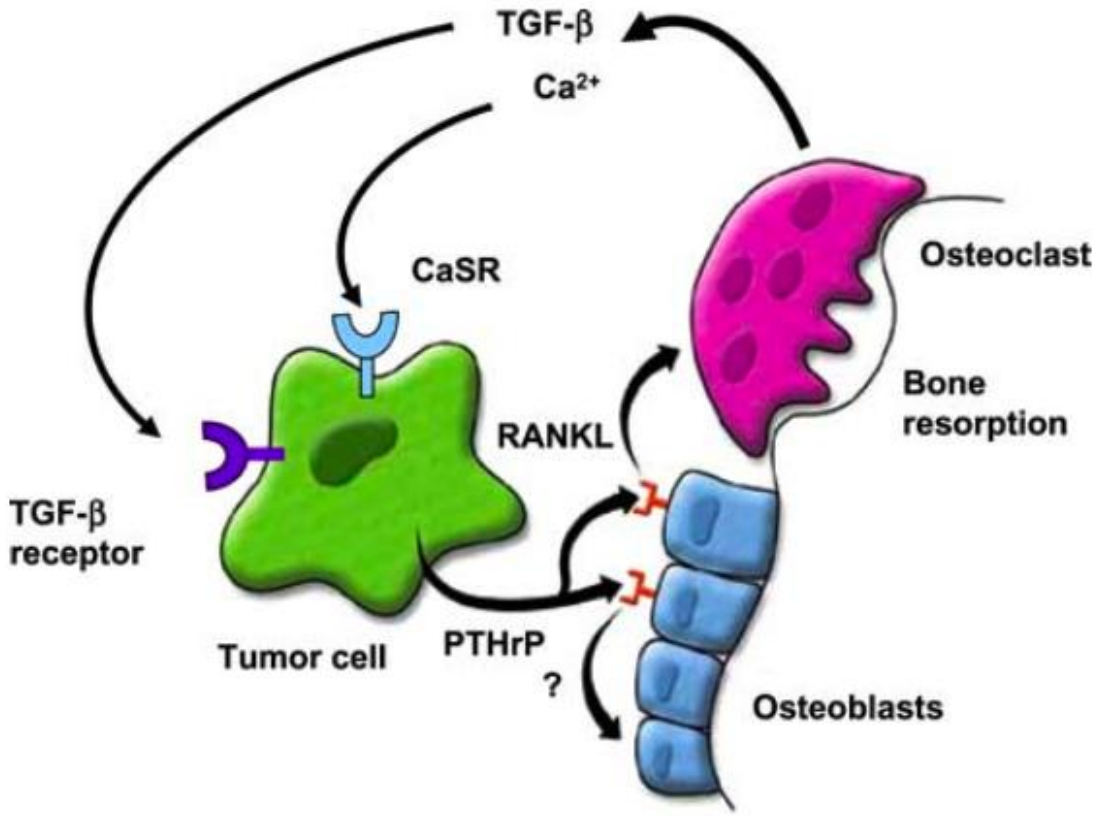


Figure 8. Vicious cycle of bone metastasis. Tumor cells secrete PTHr-P which stimulates bone resorption via RANKL expression in osteoblastic cells. Bone resorption results in release of growth factors such as TGFβ and calcium from the extracellular matrix. Calcium and TGFβ both feedback to tumor cells to increase PTHr-P production that amplifies favorable signals for tumor localization in bone [146].

ephrinA1 in bone metastasis [147]. Likewise studies have implicated A class receptors in giant cell tumors [148], and prostate cancer metastasis [149].

Cytokine Signaling and Growth Factors in Bone Metastasis

The formation of bone metastasis requires significant alteration of the balance between bone formation and bone resorption. In many cases this imbalance is achieved through tumor cell production of hormones, growth factors, chemokines or cytokines that contribute to further propagation of aberrant behavior by bone cells. Some of these factors are: TNFα, PTHrP, PGE2, IL-1, IL-6, IL-8, IL-11, IL-15, IL-17, LIF, and Oncostatin M [150-152]. TNFα is expressed primarily by activated macrophages and directly regulates recruitment of osteoclast precursors to sites of resorption. PTHrP is an osteoclast activating factor and its overproduction has been documented in skeletal metastases from breast cancer versus non-skeletal metastases from breast cancer [146]. Stromal components are responsible for the increase in PTHrP levels and the higher levels allow binding of its receptors on osteoblasts, which in response secrete RANKL and MCSF resulting in more osteoclast differentiation and bone resorption. Many of the interleukins under study for roles in breast cancer and bone metastasis are pro-inflammatory cytokines. IL-1 is expressed by activated macrophages and has been shown to stimulate RANKL expression and pit formation [153]. IL-6 also contributes to the local inflammatory response but can also contribute to bone resorption by inducing production of RANKL by osteoblasts upon binding.

Further, IL-6 induces tumor cells to produce PTHrP, IL-8, and IL-11. These later interleukins, as well as IL-15 and IL-17, are also all able to induce macrophage recruitment subsequent osteoclast migration and osteoclast activation through inducing RANKL release.

Eph Receptors as Targets for Breast Cancer Therapeutics

Since Eph receptors are often overexpressed in malignant cancer and reduction of Eph receptor levels was found to be efficacious in tumor inhibition in animal models, a wide range of therapeutic strategies targeting Eph receptor has been recently developed for cancer treatment. These approaches include activating monoclonal antibodies against Eph receptors, ligand- or activating antibody cytotoxin conjugates, small interfering RNAs (siRNA), antagonistic peptides, small molecular inhibitors, and immunotherapy (Table 2).

Carles-Kinch et al. first reported that activating monoclonal antibodies against EphA2 inhibited tumor growth in soft agar and prevented tubular network formation on Matrigel [113]. Coffman et al. subsequently showed that similar anti-EphA2 agonistic antibodies selectively bind epitopes on malignant cells and decreased tumor growth in xenograft tumor models [154, 155]. The mechanism of action of these antibodies appears to mimic ephrin ligands, inducing receptor phosphorylation and subsequent internalization and degradation [113, 154, 155]. However, it is not clear if agonistic antibody-induced EphA2 receptor forward signaling also conveys an inhibitory signal to promote tumor suppression. Regardless, the ability of ephrins and anti-EphA2 antibodies to distinguish

malignant from non-malignant cells prompted the development of ligand- or agonistic antibody-toxin conjugates. Wykosky et al. reported a novel cytotoxin composed of the ephrinA1 ligand conjugated to a genetically modified bacterial toxin, Pseudomonas exotoxin A [156]. EphrinA1-conjugates exhibits potent and dose-dependent killing of cancer cells that expressing high levels of EphA2 receptor, including glioblastoma multiforme cells, as well as breast and prostate cancer cells [156]. An anti-EphA2 antibody, conjugated with the microtubule polymerization inhibitor monomethylauristatin phenylalanine (MMAF) has also been developed by MedImmune Inc. for targeted therapy. The conjugated antibody significantly inhibits tumor cell growth both *in vitro* and *in vivo* without any observable adverse effects [157]. These findings make ephrinA1- or anti-EphA2-based cytotoxins a potentially attractive therapeutic strategy for the treatment of breast cancer.

Small interfering RNAs (siRNAs) that specifically inhibit gene expression have rapidly become a powerful tool in both mechanistic studies and targeted therapeutics. It has been previously reported that siRNAs directed against EphA2 resulted in decreased protein expression decreased tumor growth in a pancreatic cancer xenograft model [89]. More recently, siRNAs against EphA2 were incorporated into packaging liposomes composed of the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) for efficient *in vivo* delivery. Neutral liposome-coupled EphA2 siRNA reduced tumor growth in an orthotopic mouse model of ovarian cancer both in the presence and absence of paclitaxel [90, 158],

suggesting the feasibility of siRNA as a clinically applicable therapeutic approach.

Eph receptors and ephrins have emerged as critical regulators of tumor angiogenesis, making them attractive targets for inhibition of neovascularization [1, 75]. More importantly, Eph/ephrin signaling provides a possible mechanism responsible for resistance to anti-VEGF therapy [104]. Soluble Eph receptors have been used to inhibit endogenous Eph receptor signaling in vascular endothelium and tumor angiogenesis in vivo [88, 110, 159, 160]. More recently, the Pasquale laboratory has developed a peptide, TNYL-RAW, which competes with ephrin-B2 for binding to EphB4 receptor [161, 162]. In addition, two isomeric small molecule compounds have been identified that selectively inhibit ephrin binding to EphA4 and EphA2 [16, 161]. Both the EphB4 blocking peptide and EphA2/EphA4 antagonistic compounds inhibit Eph receptor phosphorylation and capillary-like tube formation in human umbilical vein endothelial cells [16], suggesting that they can potentially serve as starting points to develop anti-angiogenic therapies in cancer treatment.

In addition to being direct targets for therapeutic intervention, EphA2-derived peptides have been used in a dendritic cell-based vaccine for immunotherapy in glioblastoma multiform and colon cancer [163-165]. Early studies showed that in renal cell carcinoma, EphA2-derived peptides induced specific, tumor-reactive

Table 3. Eph-Ephrin Based Therapeutics (adapted from [166, 167])

Molecules/Treatment	Target	Tumor/Tissue Type	Activity	Reference
Kinase Inhibitors	EphB4	Angiogenic targets	ATP Competition	[168-170]
Kinase Inhibitors	Eph Receptors		ATP Competition	[171-176]
siRNA	EphA2	Ovarian, Pancreatic	mRNA downregulation	[89, 90, 177]
oligonucleotides	EphA2	Breast	Protein downregulation	[113]
siRNA	EphB4	Ovarian, Colon, Prostate, Bladder, Breast	mRNA downregulation	[84, 92, 178-180]
oligonucleotides	EphB4	Prostate, Bladder, Breast, Ovarian	Protein downregulation	[84, 92, 178, 179]
EphA2-Fc and EphA3-Fc	EphrinA	Pancreatic, Breast	Eph Competition	[88, 159, 160, 181]
sEphB4	EphrinB	Head and Neck, Breast	Eph Competition	[182, 183]
KYL peptides	EphA4	Neurons	Ephrin Competition	[161, 184]
SNEW peptides	EphB2	Neurons	Ephrin Competition	[162, 185]
TNYL-RAW peptides	EphB4	Neurons	Ephrin Competition	[185-187]
Dimethyl-pyrrole derivatives	EphA2, EphA4		Ephrin Competition	[161, 188]
2H9 antagonistic mAb	EphB2	Colon	Ephrin Competition	[189]
Antibody Conjugates	EphA2	Ovarian, Breast	Eph Activation and Degradation	[113, 154, 157, 190-192]
Bispecific antibody	EphA2		T cell recruitment/redirection	[155]
Adenoviral therapy	EphA2	Pancreatic	Transduction of EphA2 Tumor Cells	[193]
EphrinA1 exotoxin	EphA Receptors	Brain	Exotoxin Internalization	[156]
EphrinA1 Fc	EphA Receptors	Breast	Eph Activation/Degradation	[194]
EphrinB2 Fc	EphB4		Eph	[109]

			Activation/Degradation	
EphrinA1 Nanoshells	EphA Receptors	Prostate	Photo-thermal ablation of tumor cells via absorption	[195]
^{64}Cu-DOTA-1C1mAb	EphA2	Colon, Prostate, Ovarian, Melanoma	RadioimmunoPET	[196]
YSA-peptide-magnetic nanoparticles	EphA2	Ovarian, Leukemia	Binding for cell capture	[197, 198]
^{111}Indium-labeled antibody	EphA3	Melanoma	Binding for tumor detection	[199]

CD8+ and CD4+ T cell responses. The reactivity of CD8+ T cells to EphA2 peptides was stronger in T cells isolated from post-surgery disease-free patients than from patients with active disease, suggesting that the immune system of cancer patients actively monitors EphA2-derived epitopes [163]. More recently, vaccination using dendritic cells pulsed with EphA2 peptides in a murine colon cancer model revealed that immunization inhibited the growth of MC38 tumors expressing EphA2, but did not have an effect on BL6 tumors that do not express EphA2 [164]. Furthermore, Hatano et al. reported that stimulation of peripheral blood mononuclear cells from glioma patients and control healthy donors with dendritic cells loaded with EphA2 peptide elicited an antigen specific cytotoxic T cell response [165]. These preliminary results demonstrate that EphA2-derived epitopes may represent important candidate vaccines to be tested in clinical trials for the treatment of malignant cancers.

Conclusion

The field of Eph receptors and their interacting ligands (ephrins) is relatively young, having only been active for the past 20 years. However, the field of Eph biology has grown immensely in recent years. It has grown beyond the roles in normal physiology during embryonic development and axonal guidance to encompass propagating disease pathogenesis (e.g. tumorigenesis) and maintaining intricate cell communication signals between cells. Research from our lab and others has greatly expanded the evidence implicating Eph and ephrin signaling in cancer and tumor progression while also expanding the known

functions of Eph receptors and their respective ligands. As this knowledge has expanded, however, so has the complexity and paradoxical effects of Ephs and ephrins. An example of this is demonstrated through multiple screening methods showing that while human cancers express multiple Ephs and/or ephrins, both increased and decreased expression can lead to tumorigenesis and/or tumor progression. Furthermore, as demonstrated recently in elegant work by Bing Cheng Wang's group the same signaling pathway can be modulated in the same cancer leading to different outcomes dependent on whether or not the ephrin ligand is present [37]. Consistent with this is the evidence generated confirming the ability of Eph receptors and ephrin ligands to both promote and inhibit tumorigenicity. The molecular mechanisms that regulate these divergent functions, as well as the specific contexts under which tumor promoting versus tumor suppressive functions are selected, are still being studied and only now being brought to light.

Eph receptors are only now being seen as master regulators capable of propagating oncogenic signals or attenuating them. This contrast is likely due to the ability of Eph receptors to signal in a bidirectional manner. Furthermore we have recently learned of the ability of other proto-oncogenic receptor tyrosine kinases to cooperate with Eph and ephrin signaling to influence cancer cell activity. The differences in spatial and temporal coordination of inputs, as expressed through proximity, may also result in the observed differences in Eph and ephrin signaling outcomes [200]. It is clear that a large number of cell-type-dependent and context-dependent factors contribute to the multi-faceted role of

Eph receptor in cancer cells, tumor microenvironment and homeostasis/development. As a result of this inherent complexity, Eph receptor-based therapeutic strategies must be carefully evaluated before administration. In particular, therapies designed to either activate or block an Eph receptor may also alter the signaling function of the ligand in adjacent cells, due to bidirectional signaling of the Eph/ephrin system. Further research in dissecting context-dependent Eph receptor signaling is essential for developing successful therapeutic strategies for a reliable treatment against breast cancer.

Purpose of this study

Although a great deal of information has been disseminated in regards to Eph receptors and ephrins in development and cancer, there still remains a great deal that is not understood such as: Eph receptor function in crosstalk with oncogenic pathways, microenvironmental communication, tissue/context specific interactions leading to changes in signaling, as well as how the Eph system influences the metastatic cascade from tissue invasion to metastatic cell interactions at secondary sites of colonization. This dissertation examines the function of EphA2 in normal mammary gland development to aid in understanding the balance of Eph function between normal and aberrant/oncogenic signaling. This study demonstrates that EphA2 deficiency impairs mammary gland epithelial growth and branching through a RhoA-dependent mechanism. Understanding the role of EphA2 in normal mammary gland development, and knowing that metastatic breast cancer cells have high

levels of EphA2, the second portion of my thesis focuses on how metastatic cells communicate with bone cells. We hypothesize high levels of EphA2 on breast cancer cells are able to interact with osteoclasts in the bone causing activation of these cells to induce osteolysis, a common effect seen clinically in aggressive breast cancer. Together, these data demonstrate that EphA2 regulates several processes in tumor progression, including early tumor epithelial growth and invasion, as well as metastatic progression and pathogenesis in the bone microenvironment. Thus, in elucidating these diverse roles in breast cancer, we have identified several points at which anti-EphA2 therapeutic antibody maybe an effective new therapy in the treatment of bone metastatic disease.

CHAPTER II

Regulation of mammary gland branching morphogenesis by EphA2 receptor tyrosine kinase

The work presented in this chapter was published under the same name in the journal *Molecular Biology of the Cell* May, 2009 [69]

Abstract

Eph receptor tyrosine kinases, including EphA2, are expressed in the mammary gland. However, their role in mammary gland development remains poorly understood. Using EphA2-deficient animals, we demonstrate for the first time that EphA2 receptor function is required for mammary epithelial growth and branching morphogenesis. Loss of EphA2 decreased penetration of mammary epithelium into fat pad, reduced epithelial proliferation, and inhibited epithelial branching. These defects appear to be intrinsic to loss of EphA2 in epithelium, as transplantation of EphA2-deficient mammary tissue into wild-type recipient stroma recapitulated these defects. In addition, HGF-induced mammary epithelial branching morphogenesis was significantly reduced in EphA2-deficient cells relative to wild-type cells, which correlated with elevated basal RhoA activity. Moreover, inhibition of ROCK kinase activity in EphA2-deficient mammary epithelium rescued branching defects in primary cell and organoid cultures. These results suggest that EphA2 receptor acts as a positive regulator

in mammary gland development, functioning downstream of HGF to regulate branching through inhibition of RhoA. Together, these data demonstrate a positive role for EphA2 during normal mammary epithelial proliferation and branching morphogenesis.

Introduction

Mammary epithelial morphogenesis is a complex developmental process during which an extensive network of branched ducts forms from a rudimentary epithelial bud [reviewed in [64, 201]]. This process, termed branching morphogenesis, is most active during puberty. In response to hormonal stimuli, terminal end buds (TEB) form at the tips of the ducts and invade into the surrounding stroma. New primary ducts then form by bifurcation of the TEBs and secondary side-branches sprout laterally from the trailing ducts. This process is reiterated through branching and tissue remodeling until the entire mammary fat pad is filled with a ductal tree in the virgin gland. During pregnancy, the mammary epithelium undergoes differentiation and expands drastically to meet the demand of milk production throughout lactation. After weaning, the mammary epithelium regresses through a process of programmed cell death.

Mammary gland branching morphogenesis is regulated by endocrine hormones and local paracrine interaction between the developing epithelial ducts and their adjacent mesenchymal stroma. Although the mediators of the complex interaction in mammary gland development are not fully characterized, receptor tyrosine kinases (RTK) are among the critical regulators of branching

morphogenesis [57]. Hepatocyte growth factor/scatter factor (HGF/SF), a mesenchymal derived mitogen and morphogen, induces branching morphogenesis through its receptor c-Met, which is expressed on mammary epithelial cells [reviewed in [202, 203]]. More recently, expression of multiple Eph family RTKs has been reported in the mammary gland [reviewed in [204]]. However, their role in branching morphogenesis remains to be investigated.

The Eph RTK family is the largest family of RTKs identified in the genome, with at least 15 receptors and 9 ligands identified in vertebrates [Reviewed in [75, 184]]. The family is subdivided into class A and class B based on homology and binding affinity for two distinct types of membrane-anchored ephrin ligands. Class B receptors generally bind to class B ephrins that are attached to the cell membrane by a transmembrane-spanning domain, while A class receptors normally interact with glycosyl-phosphatidylinositol (GPI)-linked class A ephrins, although interclass binding does occur among certain family members [Reviewed in [205, 206]]. These molecules function in cell-cell communication during embryogenesis to regulate angiogenic remodeling processes, axon guidance, and tissue boundary formation [Reviewed in [18, 207]]. In adult organisms, members of this RTK family have been linked to tumor progression and neovascularization [Reviewed in [206]].

The first Eph receptors discovered in mammary gland are EphB4 (myk-1) and EphA2 (myk-2) [68]. The EphB4 receptor is expressed predominantly on myoepithelial cells surrounding the ducts and alveoli, whereas its cognate ligand, ephrin-B2, is expressed complementarily in luminal epithelial cells. Expression of

both ligand and receptor is estrogen-dependent [65]. More recently, a genome-wide transcript analysis identified EphA2 receptor and ephrin-B1 as the only two Eph molecules that are enriched in TEBs relative to ducts [67]. Functionally, overexpression of EphB4 in mammary epithelial cells in MMTV-EphB4 transgenic mice disrupts the patterning of the normal mammary ductal tree, induces angiogenesis, accelerates tumor formation, and promotes metastasis when co-expressed with MMTV-Neu in bigenic mice [66]. EphA2 receptor overexpression has been associated with many types of cancer, including breast cancer [reviewed in [74, 75, 208]]. In addition, EphA2 has been shown to regulate HGF-induced MDCK cell branching morphogenesis in three-dimensional collagen gels [53]. However, the role of EphA2 in mammary gland development remains unknown.

In this study we investigated the role of EphA2 in mammary gland branching morphogenesis in EphA2-deficient mice. We found that loss of EphA2 inhibits the proliferation of the mammary epithelium and delays ductal branching necessary for complete fat pad filling. At the cellular level, EphA2-deficiency resulted in marked reduction of branching in response to HGF stimulation. This defect, at least in part, is due to misregulation of Rho family GTPase function. These results suggest that EphA2 is required for mammary gland branching morphogenesis in vivo. Taken together, these data demonstrate a positive role for EphA2 in proliferation and branching morphogenesis of normal mammary epithelium.

Materials and Methods

Animals: Animals were housed under pathogen-free conditions, and experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. EphA2-deficient mice were backcrossed with FVB animals for 7 to 10 generations prior to analysis. Animals that were wild-type, heterozygous, or null for *ephA2* [105, 106] were identified by polymerase chain reaction (PCR) analysis of genomic DNA from tail biopsy using the following primers: 5'-GGG TGC CAA AGT AGA ACT GCG-3' (forward), 5'-GAC AGA ATA AAA CGC ACG GGT G-3' (neo), 5'-TTC AGC CAA GCC TAT GTA GAA AGC-3' (reverse).

Reagents: Antibodies used include anti-EphA2 (SC-924 2 mg/ml, Santa Cruz Biotechnology; Santa Cruz, CA; 5 mg/ml, Zymed Laboratories, Burlingame, CA; D7 clone 2 mg/ml, Upstate Biotechnology, Lake Placid, NY), anti-Ephrin-A1 (clone P1 1:200, Immunex, Seattle, WA), normal rabbit IgG (Santa Cruz Biotechnology), anti-b-tubulin (1:500, Sigma-Aldrich, St. Louis, MO), biotinylated anti-PCNA (1:500, BD Biosciences, San Jose, CA). Avidin peroxidase (ABC) reagents were purchased from Molecular Probes (Eugene, OR). 4',6-Diamidino-2 phenylindole dihydrochloride (DAPI) was purchased from Sigma-Aldrich. Liquid 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit was purchased from Zymed Laboratories. Recombinant murine HGF was purchased from R&D Systems (Minneapolis, MN).

Whole Mount Mammary Gland Analyses: Whole-mount hematoxylin staining of mammary glands was performed by taking number 4 inguinal mammary glands and fixing in 10% buffered formalin (Fisher, SF93-4) overnight at 4°C. The glands were washed in acetone, equilibrated into 100% ethanol, and stained in Mayer's hematoxylin solution (VWR Scientific, West Chester, PA) for one hour at room temperature, light protected. Following staining the glands were destained in tap water and then further destained in 50% ethanol acidified with hydrochloric acid at a 0.05 M final concentration. The glands were then dehydrated in a graded ethanol series followed by xylenes, and mounted on slides for photodocumentation.

Proliferation, apoptosis, and immunohistochemistry assays: Proliferation and apoptosis in mammary gland in situ were assessed by PNCA immunohistochemistry or TUNEL analysis, as previously described [5]. For immunohistochemistry, sections were de-waxed, rehydrated, and subjected to thermal antigen retrieval in citrate buffer (2 mM citric acid, 10 mM sodium citrate, pH 6.0) using a PickCell Laboratories 2100 Retriever as per manufacturer's instructions. Sections were incubated with primary anti-EphA2 antibody (5 mg/ml, Zymed Laboratories), anti-ephrin-A1 antibody (1:200, Immunex), or control rabbit IgG (5 mg/ml, Santa Cruz Biotechnology) overnight at 4°C, followed by 1 hour room temperature incubation with biotinylated anti-rabbit antibodies (BD Biosciences). Sections were then treated incubated with avidin-peroxidase

(Vector Laboratories), followed by DAB substrate, counterstained with hematoxylin (Fisher Scientific) and mounted.

Mammary Fat Pad Clearing and Transplantation: Fat pad clearing and transplantation of EphA2-deficient mammary tissue into wild-type hosts, as well as wild-type tissue into EphA2-deficient hosts, was performed as described previously [209]. Briefly, the endogenous epithelium was surgically removed from the right #4 inguinal mammary gland of 3-week old mice by excising the portion of the fat pad between the nipple and the lymph node, which contains the endogenous epithelium rudiment. A small portion (approximately 2 mm^2) of donor tissue from 6-week old female mammary glands was engrafted into the remaining fat pad. Mammary glands harboring transplanted tissue were harvested 8 weeks post-transplantation and processed for whole-mount staining as described above. Engraftment of exogenous mammary epithelium was verified by radial outgrowth of epithelium, versus glands contaminated with endogenous epithelium that grows directionally from the nipple toward the body cavity.

Isolation and Culture of Primary Mammary Epithelial Cells: Primary mouse mammary epithelial cells (PMEC) were isolated from FVB wildtype and EphA2 deficient FVB female mice and cultured as follows: mammary glands were collected under sterile conditions and digested at 37°C for 4 h in 3 mg/ml collagenase A (Beohringer Mannheim #103578) in PBS (pH 7.4), 100 units/ml of

hyalurondidase (Sigma #H-4272), and 1:1000 dilution of Fungizone (Invitrogen #15290-018). The cell suspension was first plated on bacterial Petri dishes for 3 to 5 hours to separate epithelial cells from fibroblasts, which adhere to Petri dishes. The epithelium-enriched cell suspension was then plated on dishes coated with collagen (Vitrogen) in 0.02N acetic acid washed with PBS before addition of cell culture media consisting of serum-free DMEM-F12 (50:50; Gibco BRL), 5 ng/ml estrogen (Sigma E-4389), 1 ng/ml progesterone (Sigma P-7556), 5 ng/ml EGF (Sigma E-4127), and 5 mg/ml insulin (Sigma I-1882) and cultured at 37°C in 5% CO_2.

In vitro branching morphogenesis assays: HGF-induced branching of PMECs isolated from wild-type or EphA2-deficient female mice was scored in three-dimensional mammosphere culture using a modification of previously described methods [210]. Briefly, PMECs were trypsinized and 100,000 cells were plated on a thin layer of growth factor-reduced Matrigel (BD Biosciences) in 8 well chamber slides (BD Falcon). Cultures were maintained in normal PMEC media supplemented with 2% growth factor-reduced Matrigel and 5% FCS in the presence or absence of 20 ng/ml recombinant murine HGF (R&D Systems) for 5 days, changing the media after 48 hours. Cultures were photographed on day 5 using an Olympus CK40 inverted microscope with digital camera, and branching was scored by counting branches in 4 independent 10X photographs per culture condition. For some experiments, PMECs were infected with 10^8 pfu/ml recombinant adenoviruses harboring constitutively active RhoA (Q63L, Cell

Biolabs), EphA2, or control b-galactosidase [5] 24 hours prior to mammosphere culture. For ROCK inhibition studies, PMECs were pre-treated with 0, 0.1 mM, 1 mM, or 10 mM Y27632 ROCK inhibitor (Calbiochem) or vehicle control for 1 hour prior to mammosphere culture, and cultures were treated again after 48 hours for 1 hour. All experiments were performed three times with 4 independent cultures per condition in each experiment.

Rho Kinase Assay: Primary mammary epithelial cells were plated in 6 well plates and stimulated with HGF (25 ng/ml) for 15 minutes upon reaching ~60% confluency. Cells were harvested in a 1.0% Triton X-100 lysis buffer consisting of 50mM Tris-HCl, pH 8.0, 1.0% Triton X-100, 1 mM PMSF, 5 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mM sodium orthovanadate. Harvested cells were sonicated and cleared lysates were assayed for ROCK activity using the CycLex Rho-Kinase Assay Kit (MBL Inc.) according to manufacturer's protocol. Briefly, 10 µL of each sample was incubated with a substrate corresponding to the C terminus of recombinant Myosin Binding Subunit of myosin phosphatase (MBS). The phosphorylated form of threonine 696 of the MBS was detected by an HRP conjugated detection antibody AF20, coupled with TMB color reaction. Absorbance was measured at 450nm, which reflects relative amount of Rho-kinase activity. For some experiments, PMECs were infected with 10^8 pfu/ml recombinant adenoviruses harboring EphA2, or control b-galactosidase [5] 48 hours prior the ROCK assay.

In Situ Rho/Rac Activity Detection Assays: Detection of GTP-bound Rho and Rac in mammary gland sections was performed as described previously [211]. Briefly, paraffin-embedded tumor sections were rehydrated, treated with 0.01% trypsin (Cellgro/Mediatech) for 5 minutes, and blocked for 30 minutes in M.O.M. dilutent (Molecular Probes). Sections were incubated with purified Pak-1 binding domain-glutathione S-transferase (PBD-GST), Rhotekin binding domain GST (RBD-GST) fusion protein, or GST control in M.O.M. dilutent containing 10 mM $MgCl_2$ for 15 minutes at room temperature. Binding of GST fusion proteins was detected using an anti-GST antibody (Santa Cruz Biotechnology) followed by anti-rabbit Cy3. Sections were counterstained with DAPI, photographed, and binding of GST fusion proteins quantified based on Cy3+ pixel area using NIH Image J software.

Statistical Analyses: Statistical analysis of developmental and in vitro studies were performed using 2-tailed, paired student's T-tests. For tumor studies, both T-tests and Chi square analyses were performed.

Results

Loss of EphA2 impairs normal development and architecture of the mammary epithelial tree. We first assessed expression of EphA2 and its preferred ligand, ephrin-A1, in normal mammary gland tissue isolated from 6 week old female mice. We observed EphA2 expression on the surface of luminal epithelial cells, while the surrounding myoepithelium and stroma did not express detectable protein under our staining conditions (Fig. 9A). We confirmed

specificity of staining, as well as loss of EphA2 expression in EphA2-deficient animals, by probing mammary gland tissue sections (Fig. 9A) and mammary gland lysates (Fig. 9B) with anti-EphA2 antibodies. EphrinA1 protein expression patterns were similar to those observed for EphA2, with protein detected on the surface of luminal epithelial cells (Fig. 9C). These expression patterns are consistent with reported mRNA expression of EphA2 and ephrinA1 in luminal epithelial cells [67], suggesting that this receptor-ligand pair may regulate epithelial morphogenesis.

To determine the role of EphA2 in mammary gland morphogenesis, we performed whole mount analysis of mammary glands from EphA2-deficient mice and wild-type littermate controls following a time course. In control animals, at the onset of puberty at approximately 3.5 weeks, the rudimentary mammary epithelium anlagen undergoes rapid proliferation and invades the stroma by directional growth and branching, giving rise to the characteristic mammary epithelial tree that populates the entire mammary fat pad at 10 weeks of age. In contrast, the EphA2-deficient animals exhibit severe growth retardation (Fig. 10). This defect is more prominent at 5 and 6 weeks of age, with reduced branching activity and decreased numbers of TEBs (Fig. 10A-D). In addition, outgrowth of the mammary epithelial tree was also retarded in these animals (Fig. 10A & D). In mature animals at 12 weeks, approximately 50% of the EphA2-deficient animals exhibited a fully formed mammary ductal tree (Fig. 10D). Failure of the mammary epithelium to fully penetrate the mammary fat pad persists in approximately 30% of these animals at 8 months [5].

We did not observe any defects in expansion of the mammary epithelium during pregnancy, function during lactation, or in apoptosis during post-lactational involution in EphA2-deficient animals relative to controls (data not shown).

To dissect cellular mechanisms responsible for defects in outgrowth and branching in EphA2-deficient mice, we compared proliferation and apoptosis of mammary epithelial cells in these and control animals. Cell growth was assessed by quantifying expression of proliferating cell nuclear antigen (PCNA), a marker for actively dividing cells. As shown in Fig. 11, high proliferative activity

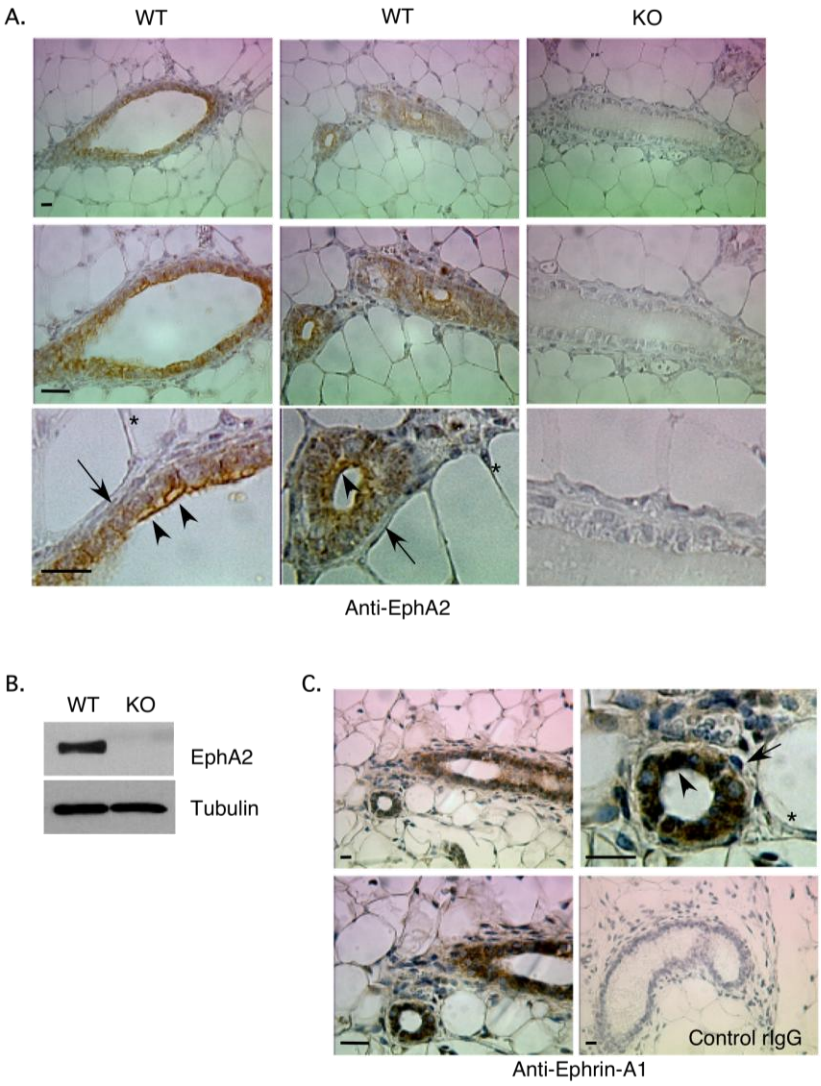


Figure 19. EphA2 receptor tyrosine kinase and ephrin-A1 ligand are expressed in luminal epithelial cells in virgin mammary gland tissue. (A) Immunohistochemical staining in virgin mammary tissue sections prepared from wild-type (WT) 6-week old virgin female mice revealed EphA2 protein expression on the surface of luminal epithelial cells (arrowheads), but no apparent expression in myoepithelial cells/fibroblasts surrounding ducts (arrows) or in fatty tissue (*). Staining specificity, as well as EphA2-deficiency, was confirmed by probing mammary tissue sections prepared from age-matched EphA2-deficient (KO) virgin female mice. (B) EphA2 protein deficiency in KO mice versus WT was also confirmed by immunoblot analysis of protein lysates prepared from whole mammary gland tissue. Uniform loading was confirmed by re-probing blots for expression of tubulin. (C) We also observed expression of ephrin-A1, the primary ligand for EphA2 receptor, in luminal epithelial cells (arrowheads), versus surrounding stromal cells (arrows) and fat (*) in tissue sections prepared from 6-week old wild-type virgin female mice. Staining specificity was validated by probing tissue with control rabbit IgG (rIgG). Scale bars = 10 mm.

was observed in the mammary epithelium of 6 weeks old control wild-type mice. In contrast, cell proliferation was significantly reduced in EphA2-deficient littermates. We observed no significant difference in epithelial content and proliferation between control and EphA2-deficient mammary glands harvested from 12 week old mature female animals, indicating that mammary epithelial growth had recovered in EphA2-deficient animals (data not shown). Nor did we detect any difference in the levels of apoptosis in mammary gland between EphA2-null animals and wild-type control littermates, either at 6 weeks or 12 weeks of age (Fig. 11C).

EphA2-deficiency inhibits HGF-induced mammary epithelial cell branching morphogenesis.

Although we did not detect expression of EphA2 in the mammary gland stroma in tissue sections (Fig. 9A), lysates from primary mammary fibroblast cultures revealed low level EphA2 expression (data not shown). Therefore, reduced mammary gland branching morphogenesis observed in EphA2-null animals could be due to either defects in mammary epithelial cells or the surrounding mesenchymal stroma. To distinguish the role of EphA2 in epithelium cells versus

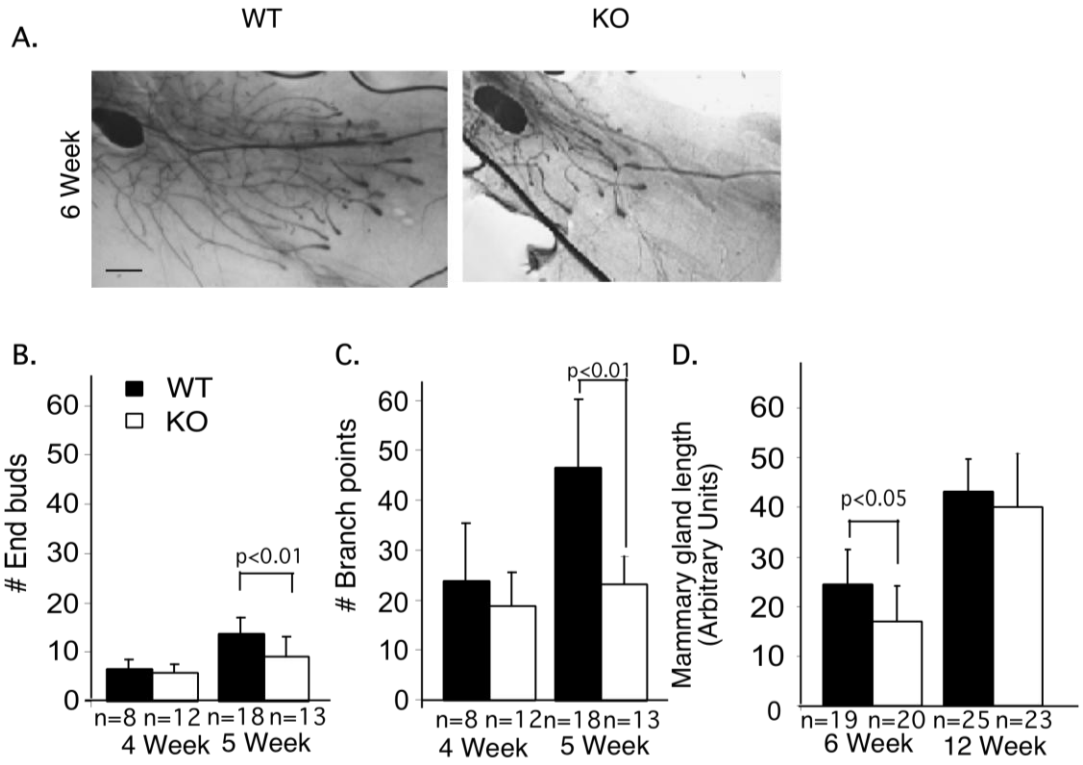


Figure 10. EphA2-deficiency impairs normal development and architecture of the mammary epithelial tree. (A) Whole-mount hematoxylin staining of number 4 inguinal mammary glands collected from WT and KO FVB female animals 5 and 6 weeks after birth. Scale bar = 200 mm. Quantification of numbers of terminal end buds (B) and branching points (C) in mammary gland whole mounts from 4-week and 5-week-old mice. (D) Mammary gland whole mounts prepared from KO EphA2 and WT EphA2 animals from 6 weeks of age and 12 weeks of age were analyzed for epithelial penetration into the fat pad by measuring the distance between the lymph node to the tips of the epithelial tree in gland. Quantification of TEBs, branching points, and the degree of epithelial penetration through the mammary fat pad was performed by analyzing whole-mount preparations from > 10 independent animals per developmental stage per genotype. Statistical analyses were performed using 2-tailed, paired student's T-tests.

stroma, we performed reciprocal transplantation experiments in which we grafted EphA2-deficient mammary gland tissue into the cleared fat pad of wild-type female mice, as well as wild-type mammary gland tissue into the cleared fat pad of EphA2-deficient female mice. Interestingly, we observed failed engraftment of EphA2-deficient mammary epithelium in 4 out of 10 animals, versus 1 out of 10 animals engrafted with wild-type epithelium. This observation is consistent with proliferation and outgrowth defects in endogenous EphA2-deficient epithelium (Figs. 10 and 11). For animals in which the donor epithelium did engraft, we observed diminished branching in wild-type animals harboring EphA2-deficient epithelium, while wild-type epithelium grafted into EphA2-deficient animals displayed robust branching 8 weeks after engraftment (Fig. 12A). These data suggest that the branching defects observed in EphA2-deficient animals are due to loss of EphA2 function in mammary epithelium versus stroma.

To validate the data derived from reciprocal transplant experiments in an in vitro system where levels of specific branching morphogens may be manipulated, we isolated primary mammary epithelial cells (PMECs) from wild-type and EphA2-deficient mice and tested their ability to branch and invade into matrix in a three-dimensional basement membrane gel. We did not observe any differences in low-level branching between wild-type and EphA2-deficient cells in untreated cultures. A number of factors acting in a paracrine fashion are known to regulate mammary gland development via branching morphogenesis [212]. In particular, HGF promotes ductal outgrowth and tubule formation in the mammary gland

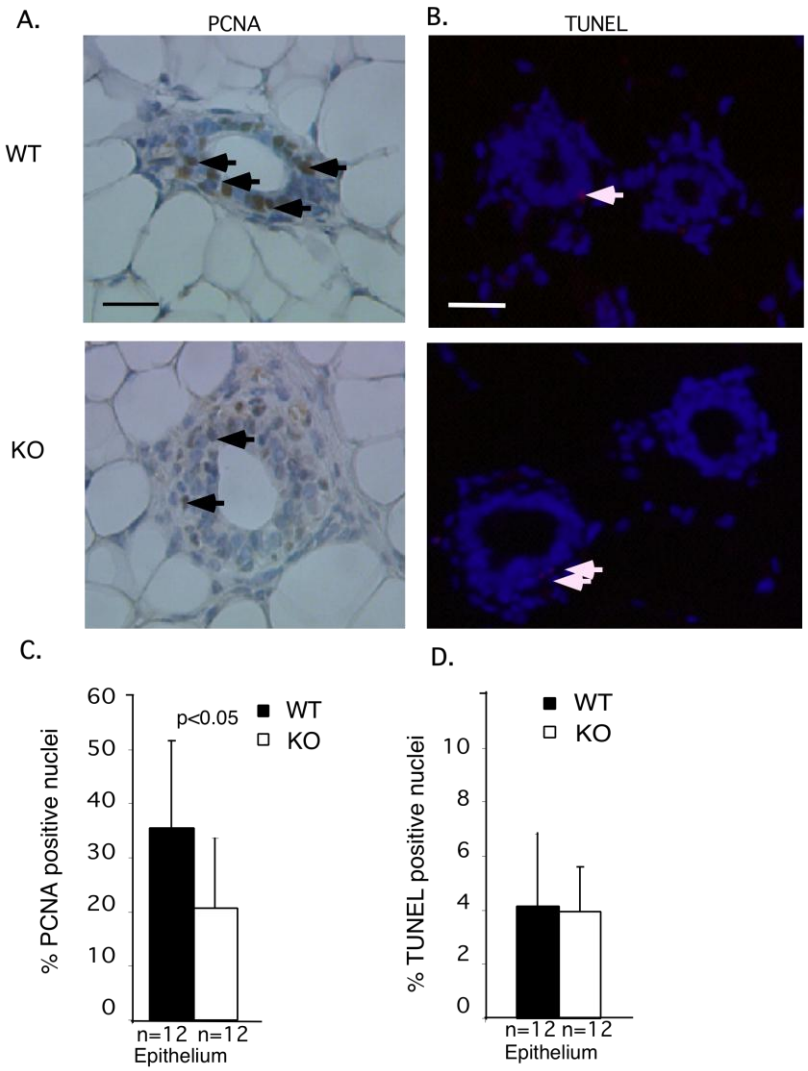


Figure 11. EphA2 deficiency inhibits proliferation but has no effect on apoptosis. (A) PCNA immunohistochemistry in mammary gland tissue sections from wild-type and EphA2-deficient animals. Arrowheads indicate PCNA+ nuclei. Scale bars = 50 mm (B) Proliferation was assessed by quantification of nuclear staining for PCNA in tissue sections. A significant reduction in the percentage of PCNA positive nuclei relative to total nuclei was observed in KO EphA2 mammary glands, compared to WT controls ($p<0.05$). (C) No significant change in the percentage of apoptotic nuclei, as assessed by TUNEL assay, was observed in KO mammary glands relative to WT controls. Quantification of the percentage of PCNA+ or TUNEL+ nuclei was performed by analyzing > 10 independent animals per developmental stage per genotype. 5 random 20X fields/tissue section were photographed for PCNA and TUNEL per each independent animal/genotype. Statistical analyses were performed using 2-tailed, paired student's T-tests.

[202]. We thus analyzed the role of EphA2 in HGF-induced mammary epithelial cell branching morphogenesis. As shown in Fig. 12, primary mammary epithelial cells form spheroid structures in growth factor reduced Matrigel, and EphA2-deficient cultures display diminished branching relative to wild-type control cells. In response to HGF stimulation, wild-type spheroids undergo extensive remodeling and branching. In contrast, EphA2-deficient spheroids fail to undergo branching morphogenesis, displaying significantly fewer branches relative to wild-type cells in response to HGF stimulation. Overexpression of wild-type EphA2 receptor in primary mammary epithelial cells via adenovirus transduction not only rescued phenotypes in EphA2-deficient cells, but also drastically enhanced branching morphogenesis in wild-type cells. Taken together, these data suggest that EphA2 receptor is required for HGF-induced branching morphogenesis.

Increased RhoA activity in EphA2-deficient cells inhibits mammary epithelial cell branching.

We next investigated the molecular mechanisms through which EphA2 regulates mammary epithelial cell branching morphogenesis. Dynamic regulation of the actin cytoskeleton is critical in a number of cellular processes including cell migration and branching morphogenesis. RhoA GTPases are key regulators of actin stress fiber formation and are necessary for cell migration [213-215]. Moreover, Ewald et al. recently reported that inhibition of ROCK kinase, a

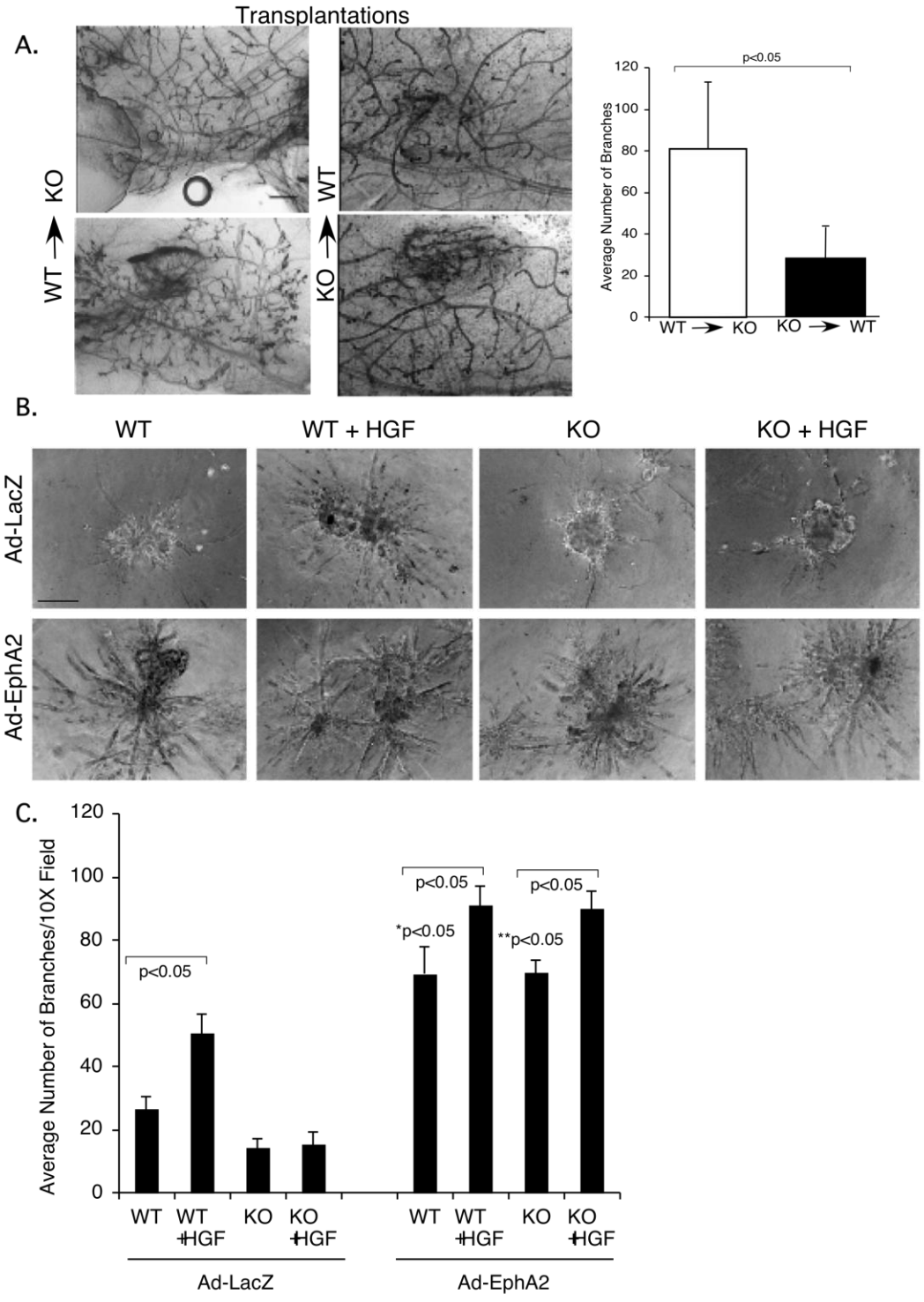

A.
Transplantations
KO
WT
WT
KO
p<0.05
Average Number of Branches
120
100
80
60
40
20
0
WT → KO
KO → WT
B.
WT
WT + HGF
KO
KO + HGF
Ad-LacZ
Ad-EphA2
C.
Average Number of Branches/10X Field
120
100
80
60
40
20
0
p<0.05
*p<0.05
p<0.05
**p<0.05
p<0.05
WT
WT +HGF
KO
KO +HGF
WT
WT +HGF
KO
KO +HGF
Ad-LacZ
Ad-EphA2

Figure 12. EphA2 activity is required in mammary epithelium for optimal branching in vivo, as well as for HGF-induced epithelial cell branching morphogenesis in Matrigel. (A) To determine if defective epithelial branching in EphA2-deficient (KO) mice was due to loss of EphA2 function in epithelium versus stroma, we transplanted KO mammary tissue into the cleared fat pads of WT female mice, as well as WT mammary tissue into the cleared fat pads of KO female mice. Photomicrographs display 2 independent wholemount preparations of mammary fat pads harboring transplanted tissue. While WT epithelium displayed robust branching in KO stroma 8-weeks after transplantation, branching of KO epithelium was significantly diminished in WT hosts ($p<0.05$; 2-tailed, paired student's T-test). Scale bar = 200 mm. Quantification of branching was performed by analyzing whole-mount preparations from 5 independent transplants/condition. (B) Primary mammary epithelial cells isolated from WT or KO animals were transduced with adenoviruses expressing EphA2 (Ad-EphA2) or control LacZ (Ad-LacZ) and plated on growth-factor reduced Matrigel with or without HGF for 5 days and photographed. HGF enhances branching morphogenesis in WT, but not KO, mammary epithelial cells. The defects in KO cells was rescued by re-expressing wild-type EphA2 receptor. Scale bar = 25 mm. (C) Branching morphogenesis was quantified by counting the number of branches per photograph in 4 independent samples per culture condition in three independent experiments. Statistical analyses were performed using 2-tailed, paired student's T-tests.

downstream effector of RhoA, results in hyperbranched mammary epithelium in organoid cultures [216], suggesting that RhoA is crucial for proper branching morphogenesis in mammary epithelial development. To investigate whether RhoA GTPase is involved in HGF/EphA2 induced branching of mammary epithelial cells, we measured ROCK kinase activity in wild-type and EphA2-deficient mammary epithelium. As shown in Fig. 13A, HGF stimulation of primary mammary epithelial cells for 15 minutes induced ROCK kinase activity in wild-type cells. Interestingly, the basal level of ROCK activity is markedly increased in EphA2-deficient cells, and this level does not change in response to HGF stimulation. Experiments in which we restored expression of EphA2 with adenoviral infection EphA2-deficient cells rescued the phenotype by reducing ROCK activity to near wild-type levels, though levels of ROCK activity remained similar in wild-type cells upon adenoviral EphA2 overexpression (Fig. 13A). To determine the level of active RhoA GTPase in vivo, we performed effector-binding assays on mammary gland tissue sections in situ [211]. Consistent with results from ROCK assay in vitro, RhoA activity is markedly increased in EphA2-deficient mammary gland epithelium in 6 week old mice, compared to that in wild-type control littermates, as judged by GST-Rhotekin binding detected by anti-GST antibodies (Fig. 13B). As a balance of RhoA and other small Rho GTPases activities (such as Rac1 and Cdc42) often determines the biological outcome in branching morphogenesis, we assayed Rac1 and Cdc42 activity in situ using

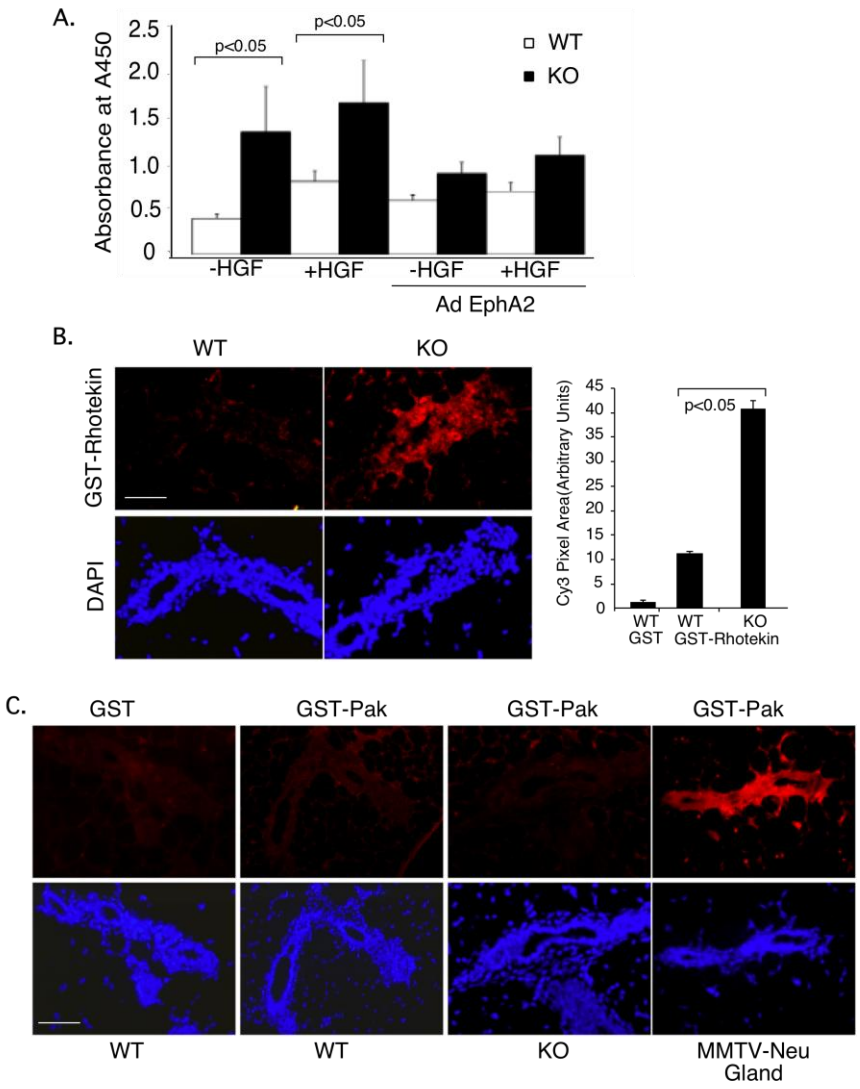


Figure 13. Increased RhoA activity in EphA2 deficient mammary epithelial cells. (A) Rho kinase activity was measured by an immune-based kinase assay, as described in Materials and Methods. Rho kinase activity was significantly increased in EphA2-deficient mammary epithelial cells ($p<0.05$; 2-tailed, paired student's T-test). Data are a representation of 3 independent experiments. (B) Rho GTPase activities were analyzed in 6 week old mammary glands in situ by incubating tissue sections with GST-Rhotekin or GST-Pak for the detection of RhoA and Rac1/Cdc42 activities, respectively; followed by detection using a Cy3 conjugated anti-GST antibody. Scale bars = 50 mm. Rho activity was quantified based on Cy3 positive pixel area using NIH image J software. Quantification of Rho activity was performed in four 40X fields/section in tissue sections from three independent wild-type or EphA2-deficient mammary animals. Statistical analyses were performed using 2-tailed, paired student's T-tests.

GST-Pak proteins. We did not detect any significant levels of Rac1 and Cdc42 activities in the 6-week-old mammary gland sections (Fig. 13C).

To determine the functional relevance of RhoA activity in EphA2-mediated branching morphogenesis, we first examined HGF-induced branching of primary mammary epithelial cells in cells expressing control adenovirus LacZ versus adenovirus harboring constitutively active RhoA mutant (CA-Rho). CA-RhoA significantly inhibited branching in wild-type cultures, and slightly reduced branching in EphA2-deficient cultures (Fig. 14A, B). We also assessed branching in the presence or absence of Y27632, an inhibitor of ROCK kinase. Treatment with Y27632 rescued branching defects in EphA2-deficient cells in both the presence and absence of HGF (Fig. 16D). HGF stimulation enhanced Y27632-mediated branching in wild-type cells in a dose-dependent manner (Fig. 14E). Interestingly, HGF did not affect Y27632-mediated branching in EphA2-deficient cells. As HGF-mediated branching is impaired in EphA2-deficient mammary epithelium (Fig. 14), and as the ROCK kinase inhibitor rescues the branching defects in EphA2-deficienct cells in either the presence or absence of HGF (Fig. 14E), these data support a model (Fig. 15) in which EphA2 functions downstream of HGF to regulate mammary epithelial branching through inhibition of RhoA.

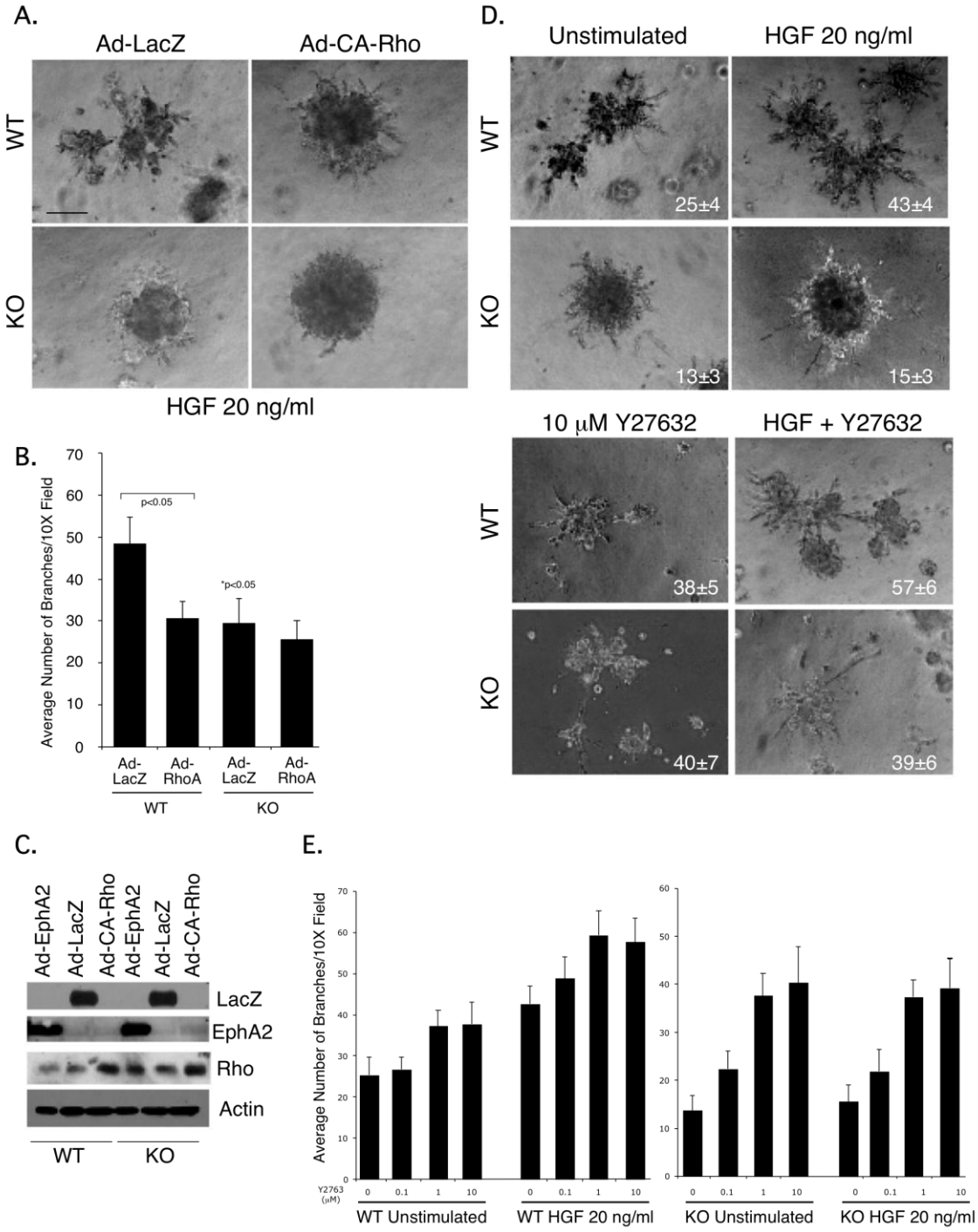

A.
Ad-LacZ
Ad-CA-Rho
WT
KO
HGF 20 ng/ml
B.
Average Number of Branches/10X Field
p<0.05
*p<0.05
Ad-LacZ
Ad-RhoA
Ad-LacZ
Ad-RhoA
WT
KO
C.
Ad-EphA2
Ad-LacZ
Ad-CA-Rho
Ad-EphA2
Ad-LacZ
Ad-CA-Rho
LacZ
EphA2
Rho
Actin
WT
KO
D.
Unstimulated
HGF 20 ng/ml
WT
KO
25±4
43±4
13±3
15±3
10 μM Y27632
HGF + Y27632
38±5
57±6
40±7
39±6
E.
Average Number of Branches/10X Field
Y2763 (μM)
WT Unstimulated
WT HGF 20 ng/ml
KO Unstimulated
KO HGF 20 ng/ml

Figure 14. EphA2-dependent branching morphogenesis is dependent on RhoA activity. (A) Primary mammary epithelial cells isolated from WT or KO animals were transduced with adenoviruses expressing a constitutively activated RhoA (CA-Rho) or control LacZ. Cells were plated on growth-factor reduced Matrigel with 20 ng/ml HGF for 5 days and photographed. Branching in KO LacZ control cells was significantly diminished relative to WT LacZ control cells in response to HGF (*$p<0.05$; 2-tailed, paired student's T-test). Expression of the Q63L RhoA inhibited HGF-induced branching morphogenesis in WT cells. Scale bar = 25 mm. (B) Branching morphogenesis was quantified by counting the number of branches per photograph in 4 independent samples per culture condition in three independent experiments. Statistical analyses were performed using 2-tailed, paired student's T-tests. (C) Expression of wild-type EphA2, CA-Rho, and b-galactosidase via adenovirus transduction was confirmed by immunoblot analysis. (D) Branching was also assessed in WT and KO primary mammary epithelial cells in the presence or absence of 20 ng/ml HGF and upon treatment with the Rho kinase inhibitor 10 mM Y27632. As observed previously, WT, but not KO, cells displayed elevated branching in response to HGF ($p<0.05$ WT untreated versus WT + HGF). Branching was elevated in both WT and KO cells in the presence of Y27632. While the addition of HGF enhanced Y27632-mediated branching in WT cells, adding HGF had no effect on Y27632-mediated branching in KO cells ($p<0.05$ WT versus WT Y27632 and WT Y27632 versus Y27632 + HGF; $p<0.05$ KO versus KO Y27632; 2-tailed, paired student's T-tests). (E) The differential effects of HGF Rho-dependent branching for WT versus KO cells was confirmed by a dose response assay in which cultures were treated with 0, 0.1 mM, 1 mM, or 10 mM Y27632 in the presence or absence of HGF.

Discussion

Expression of several Eph receptor tyrosine kinases in mammary gland has been reported [204]. However, their role in mammary gland development remains poorly understood. Using EphA2-null animals, we demonstrated for the first time that EphA2 receptor function is required for mammary gland branching morphogenesis. Loss of EphA2 resulted in decreased penetration of mammary epithelial into the fat pad, reduced proliferation of epithelial cells, and inhibition of mammary epithelial branching. In addition, HGF-induced mammary epithelial cell migration and branching morphogenesis was significantly reduced in EphA-deficient cells, compared with that in wild-type cells. These results suggest that EphA2 receptor acts as a positive regulator in mammary gland development.

Other studies, however, suggest that Eph receptors may inhibit mammary gland morphogenesis. Overexpression of EphB4 in mammary epithelium under the control of MMTV promoter/enhancer led to less branching activity, reduced alveolar buds, and a decrease in proliferation of mammary epithelial cells [66]. Although not observed in mammary epithelium, this phenotype is reminiscent of the effect of EphA receptor activation seen in MDCK cells in response to HGF-induced branching morphogenesis in collagen gels [53]. In this model, co-stimulation of MDCK cells with ephrinA1 and HGF inhibited sprouting and induced the collapse of pre-existing branches. These results suggest that high levels of Eph receptor signaling above the endogenous level in epithelial

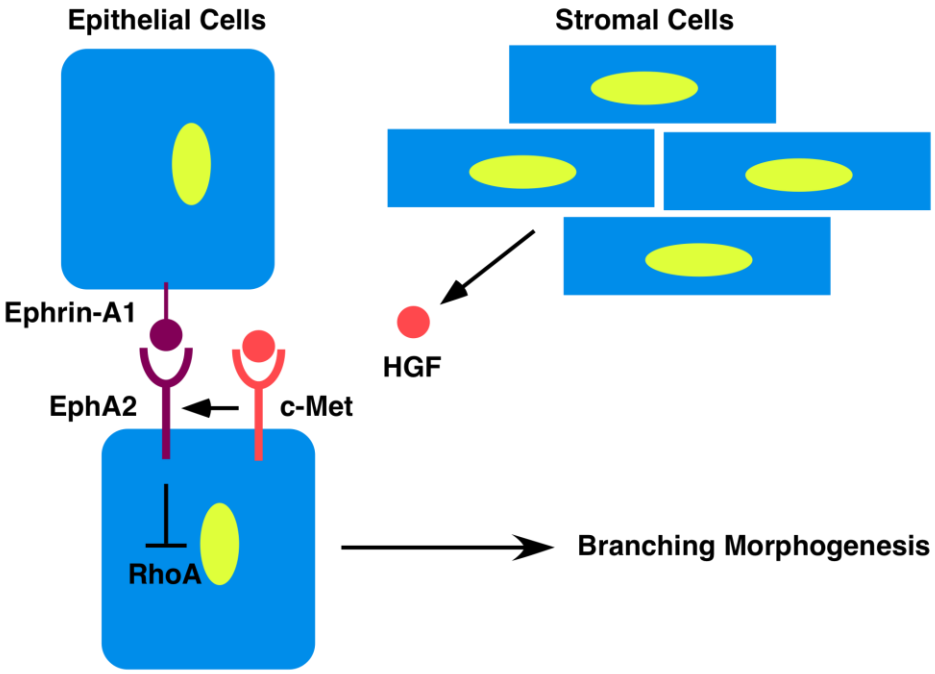


Figure 16: Model for HGF-mediated regulation of EphA2 in mammary epithelial branching morphogenesis. HGF produced by mesenchymal cells within mammary stroma binds to c-Met receptor, expressed on mammary epithelium. Through activation of EphA2 receptor function by an as yet unidentified mechanism, Rho activity is then downregulated to promote branching morphogenesis of the developing mammary epithelium during puberty.

cells, either by overexpression of receptor or exogenous stimulation with a large dose of ephrin ligand, may inhibit branching morphogenesis. However, low levels of endogenous EphA2 receptor are required for proper mammary gland development in vivo based on our data.

Epithelial branching morphogenesis is a fundamental biological process underlying the development of many organs. In breast tissue, epithelial branching morphogenesis is driven by endocrine hormonal stimuli that elicit local paracrine interactions between the developing epithelial ducts and their adjacent mesenchymal stroma. Cytokines and growth factors, such as HGF, FGF, TGF-ß, and amphiregulin are among molecules that are critical in local regulation of branching morphogenesis [57, 202, 217]. A common pathway activated downstream of these signaling molecules is the Rho family of small GTPases. These proteins cycle between an inactive, GDP-bound state and an active, GTP-bound state, regulated by guanine nucleotide exchange factors (GEFs) that exchange GDP for GTP and GTPase activating proteins (GAPs) that promote hydrolysis of GTP to GDP [213, 214]. Recently, RhoA, Rac-1, and Cdc42 GTPases have emerged as critical mediators of Eph signal transduction, as association of several Eph RTK family members with GEFs concomitant with activation of Rho family GTPases has been observed in a variety of cell types. Interactions between Eph RTKs and the GEFs ephexin, intersectin, and kalarin link Eph-mediated activation of Rho family GTPases to growth-cone collapse (ephexin) or dendritic spine morphogenesis (intersectin and kalarin) in neuronal patterning during embryogenesis [Reviewed in [18, 184, 206]]. More recently,

Eph signaling through Rho family GTPases has been linked to vascular remodeling and angiogenesis [106, 107]. In MDCK epithelial cells, Miao et al reported that ephrinA1 has no effect on RhoA but inhibits Rac1 activation induced by HGF in MDCK cells [53]. Here we show that EphA2-dependent RhoA activity modulates HGF-induced branching morphogenesis in mammary epithelial cells. First, loss of EphA2 led to a constitutive higher level of RhoA GTPase activity, which is insensitive to HGF stimulation (Fig. 13A&B). Interestingly, Rac1 activity did not appear to be significantly altered in EphA2-deficient mice, indicating that RhoA activity is a primary target regulated by the EphA2 receptor (Fig. 13C). Furthermore, a ROCK kinase inhibitor, Y27632, rescued branching defects in EphA2-deficient cells, (Fig. 14) functionally linking EphA2-dependent RhoA activity branching morphogenesis.

Regulation of Rho GTPase in mammary epithelium is complex. For example, targeted disruption of p190-B RhoGAP inhibits mammary epithelial ductal outgrowth, and heterozygous female mice display reduced proliferation within TEBs and delayed outgrowth of mammary ducts [218]. As deletion of this GAP, which negatively regulates Rho, results in elevated Rho activity, these data are consistent with our findings that elevated RhoA function in virgin mammary epithelium is associated with decreased proliferation and ductal outgrowth in EphA2-deficient mammary epithelium. Interestingly, overexpression of the same RhoGAP in mammary epithelium elevates branching in transgenic animals, though ductal elongation is still delayed [219]. These data suggest that disruption of Rho activity in mammary epithelium, either positive or negative, has

a profound impact on epithelial morphogenesis in vivo. This hypothesis is consistent with the apparent differential regulation of Rho by EphA2 signaling in normal versus transformed mammary epithelium, which is influenced by cooperative signaling pathways. In the present study, loss of EphA2 results in elevated RhoA activity in mammary epithelium that is accompanied by suppression of ductal outgrowth in vivo. Moreover, impaired HGF-mediated branching in primary mammary epithelial cells and organoid cultures derived from EphA2-deficient mice is rescued by inhibition of the downstream effector ROCK in vitro. These data are consistent with reported hyperbranching of mammary epithelial organoid cultures upon treatment with the same ROCK inhibitor [216]. In the normal mammary development, EphA2 may serve to restrict levels of active RhoA, enabling HGF to maintain levels of activity that promote the proper balance between branching and ductal outgrowth necessary for normal epithelial morphogenesis. By contrast, elevated EphA2 expression in normal mammary epithelial cells via adenoviral transduction enhances branching. These data are consistent with reports that EphA2 overexpression in non-transformed MCF10A cells confers malignant transformation and tumor forming potential in vivo [71, 113], and overexpression in 4T1 mouse mammary adenocarcinoma cells enhances tumor progression and metastasis in vivo [11]. In MMTV-Neu mice, loss of EphA2 diminishes tumorigenesis and metastasis, as well as reducing levels of active RhoA [5]. Thus, overexpression of EphA2, as well as cooperation with other oncogenic pathways in the context of mammary epithelial neoplasia, appears to enhances RhoA.

Interestingly, we did observe diminished RhoA activity in EphA2-deficient mammary epithelium relative to wild-type controls in 5 week old mice (data not shown), during which time the epithelium is still actively growing and branching. This observation suggests that EphA2 might also regulate Rho activity differentially at different stages of mammary epithelial development and/or malignant progression. For example, in normal mammary epithelial cells, ephrin-A ligands, such as ephrinA1, may engage EphA2 receptors on adjacent cells and disrupt activation of Rho GTPase, thus alleviating inhibition of epithelial branching morphogenesis. We previously reported that EphA2 physically and functionally interacts with ErbB2 and that co-expression of ErbB2 was sufficient to induce phosphorylation of EphA2 in the absence of exogenous ephrinA1 ligand. Moreover, EphA2-deficiency results in impaired Rho activation in MMTV-Neu tumors and diminished motility of primary tumor cells [5]. Cell motility in EphA2-deficient MMTV-Neu tumor cells was rescued by overexpression of activated RhoA. Thus, EphA2 receptor activation by ErbB2 and/or other receptor tyrosine kinases might enhance Rho activity, cell motility, and malignancy in the context of cancer cells in which weakened cell-cell contacts could impair interaction between EphA2 and endogenous ligands. This might also be true in 5 week old mammary glands, in which active growth might also diminish cell-cell contact and interaction between EphA2 and ephrinA1 on adjacent luminal epithelial cells. It will be of great interest to investigate differential regulation of Rho family GTPases by EphA2 in normal mammary epithelium versus mammary adenocarcinoma.

In conclusion, we provide the first evidence for EphA2 receptor regulation of normal growth and branching morphogenesis in normal mammary epithelial development. Based on reciprocal transplantation and organoid culture studies, EphA2 function specifically in mammary epithelium, rather than mesenchymal stroma, appears to mediate these processes. Our data suggest that EphA2 receptor functions downstream of HGF to regulate mammary epithelial branching through inhibition of RhoA GTPase.

CHAPTER III

REGULATION OF TUMOR INDUCED OSTEOLYSIS BY RECEPTOR TYROSINE KINASE EPHA2

Abstract

Eph receptor tyrosine kinases are membrane bound receptors often expressed in human cancers. Of the many Eph receptors, EphA2 is highly expressed in breast tumor cells and correlates with poor patient prognosis. As metastasis of breast cancer to bone is a major cause of morbidity and mortality in patients, we investigated the role of EphA2 in this clinically relevant phenomenon. Analysis of human breast-to-bone metastasis samples revealed EphA2 positive staining on tumor cells in close proximity to osteoclast at the tumor-bone interface. To define the role of EphA2 in tumor cell-host bone cell interactions, mouse tibias were injected with osteolytic breast tumor cells lacking EphA2 activity. Our data showed that inhibition of EphA2 activity significantly decreased tumor-induced osteolysis compared to controls. Further *in vitro* analysis revealed that blocking EphA2 function resulted in defective precursor maturation into functional osteoclasts. A human antibody targeted against EphA2, decreased breast tumor induced osteolysis in vivo. In summary, we propose that EphA2 regulates cell-cell interactions between tumor cells and bone cells via physical and indirect communication with osteoclasts. Our studies indicate the selective inhibition of

EphA2 at the tumor-bone interface may be a benefit for the treatment of breast-to-bone metastases.

Introduction

Metastasis to bone is a common occurrence among late stage breast cancer patients [114]. Bone metastases arising from primary breast cancers are predominately osteolytic in nature and cause skeletal complications including fractures, nerve compression, bone pain, and hypercalcemia [118, 220]. The establishment and growth of these metastases depends on the interaction between tumor cells and the host microenvironment. The metastatic cells are able to seize control of normal bone remodeling processes to induce aberrant activation of osteoclasts leading to an increase in lysis of the bone [114, 220]. Elevated osteoclast activity induces release of growth factors sequestered in the bone matrix such as transforming growth factorβ (TGF-β), calcium, fibroblast growth factor (FGF) and insulin like growth factors (IGF). These growth factors in turn promote tumor cell survival and growth in the bone microenvironment. Subsequent release of factors from the tumor cells like PTHrP (parathyroid hormone related protein), IL-1 (interleukin 1) and IL-8 (interleukin 8) can feed back to osteoblasts causing release of RANKL (receptor and activator of nuclear factorκ B ligand) and further activating osteoclasts thereby completing the "vicious cycle" of tumor induced osteolysis [114]. A critical component in tumor-induced osteolysis is the osteoclasts, large multinucleated differentiated cells with the unique ability to resorb mineralized bone [130]. Understanding the

mechanisms behind osteoclasts recruitment, maturation, and activation is key for developing new therapies to target osteolytic lesions resulting from breast cancer metastasis to the bone, which are often resistant to current therapies

Ephrin ligands and their receptors (Eph) belong to the largest family of receptor tyrosine kinases. The Eph family of receptors and ligands plays critical roles in neuronal, vascular, and intestinal development as well as cellular migration and bone morphogenesis [70, 143]. Both ephrin ligands and Eph receptors are membrane bound proteins, which signal via cell-cell contact in both the receptor and ligand expressing cells (bidirectional signaling). The family is subdivided into two subclasses based on sequence homology, binding affinity, and structure of the ephrin ligand. The A-subclass of receptors (EphA1-EphA10) bind to the ligands tethered to the cell membrane by a glycosylphosphatidlinositol (GPI) anchor (ephrinA1-ephrinA6), while the B-subclass (EphB1-EphB4, EphB6) bind to ligands containing a transmembrane domain followed by a short cytoplasmic region (ephrinB1-ephrinB3). The importance of signaling by both the receptor and ligand has been confirmed in multiple studies investigating angiogenesis, tissue boundary formation, cell sorting, and axonal guidance [221].

Recent studies demonstrated that bidirectional signaling by Eph-ephrin molecules plays an important role in bone biology. EphrinB2 expression on osteoclasts inhibits osteoclast differentiation. In contrast EphB4 expression on osteoblasts promotes differentiation [143]. This finding compliments the observed requirement for ephrinB1 ligand in the patterning of the developing skeleton [138, 139]. Class A Eph receptors have also recently been implicated in

bone homeostasis. Irie et. al. reported that ephrinA2-EphA2-mediated interaction between osteoclast precursors and osteoblasts enhances osteoclastogenesis while inhibiting osteoblast differentiation [145]. Likewise, other studies have implicated A class receptors in giant cell tumors [148], and prostate cancer metastasis to bone [147, 149]. Although Ephs have been studied in normal bone homeostasis, the role of tumor induced-osteolysis remains unclear. Here we demonstrate that breast cancer cell expression of EphA2 promotes osteoclast activation and development of osteolytic bone disease in a tumor context. Moreover, targeting EphA2 with a therapeutic, activating antibody that reduces cell surface expression and function significantly impaired breast tumor cell growth and osteolysis *in vivo*. These data provide a strong rationale for development and application of molecularly targeted therapies against EphA2 for the treatment of breast cancer bone metastatic disease.

Materials and Methods

Reagents. All experiments involving animals were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. 4T1ΔC cells were previously generated [11]. All reagents were obtained from Sigma Aldrich unless otherwise noted.

Intratibial injection. 4T1 WT and 4T1ΔC tumor cells (10^6) in a 50 μL volume of sterile PBS were injected into the left tibia of deeply anesthetized Balb/c animals of 6-8 weeks of age. The contralateral tibia was injected with 50 μL volume of

PBS alone and treated as the sham-injected control. Mice were sacrificed 10 days post surgery, and both the tumor-injected and contralateral tibias were collected for histological analysis. Similarly, the same number/volume of MDA-MB-231 cells were injected into anesthetized immunocompromised 6 week old nude female mice (Harlan Sprague Dawley). Tibias from both the tumor injected and sham contralateral legs were also harvested for analysis at 2 months post implantation. All animal studies were repeated at least twice for a total of 8 animals per condition.

Treatment with therapeutic anti-EphA2 antibody. Beginning 72 hours prior to tumor cell injection, recipient mice received intraperitoneal injections of 3F2-3M anti-EphA2 antibody (MedImmune/Astra Zeneca) or control IgG (10 mg/kg every 72 hours for 8 weeks), prior to collection and analysis of hindlimbs. At least 8 animals/condition were analyzed in 2 independent experiments

Histology. *TRAP staining*: tumor and sham injected tibias were fixed for 4 hours in fresh 4% paraformaldehyde and decalcified for 3 weeks in 14% EDTA at pH 7.4 with changes of solution every two to three days. Tissues were embedded in paraffin, and 5 μm sections were prepared for staining. For tartrate-resistant acid phosphatase (TRAP) staining the following technique was used. Sections were rehydrated through a series of ethanols and then rinsed in PBS. Following deparaffinization, histological samples were placed in warm incubation buffer (acetate buffer with tartaric acid pH 4.9) supplemented with a napthol AS-Bl

phosphate in ether substrate. After incubation of slides for 30 minutes at 42°C, freshly prepared sodium nitrite solution was mixed with pararonsaniline dye stock in basic incubation solution to produce color after 10 minutes at room temperature. Harris's acid hematoxylin was used for counterstain.

EphA2 staining. Immunohistochemical detection of EphA2 in paraffin sections from collected hindlimbs was performed as described previously [69] using a rabbit polyclonal antibody (Zymed Laboratories, 5 µg/ml, overnight at 4°C). Antigen retrieval was performed by heating in a Pickcell 2100 retriever in the presence of citrate buffer (2 mM citric acid, 10 mM sodium citrate buffer, pH 6.0). Sections were washed in PBS and incubated with primary antibody overnight, followed by biotinylated anti-rabbit IgG secondary antibody (1:200; Transduction Laboratories, BD Biosciences PharMingen) for 1 h at room temperature. Specific staining was detected using avidin-peroxidase (ABC kit, Vector Laboratories, Burlingame, CA) followed by 3,3' Diaminobenzidine (DAB) substrate (Zymed Laboratories). Sections were counterstained with hematoxylin.

Micro-computed tomography and histomorphometry. Micro computer tomography (µCT) scanning (Concorde) of osteolytic lesions using segmentation analysis (Amira) were performed at week 8 post-transplantation. Animals were sacrificed at week 8 based upon µCT scan analysis. After sacrifice, the samples fixed for 4 hours in fresh 4% paraformaldehyde and decalcified for 3 weeks in 14% EDTA at pH 7.4 with changes of solution every two to three days. Tissues were embedded in paraffin, and 5 µm sections were prepared for staining.

Histomorphometry was calculated by using 2 non-serial sections of tumor bearing limbs stained with H and E to assess BV/TV and/or with TRAP to provide osteoclast number per millimeter of bone at the tumor bone interface.

Bone marrow cell isolation. Bone marrow cells were isolated from FVB mice and were cultured for three days in α-minimal essential medium (αMEM) containing 10% fetal bovine serum and 10ng/ml of recombinant MCSF (Peprotech). Following, three days of culture non-adherent cells were removed via PBS wash leaving adherent primary bone marrow cells. These primary cells were further differentiated to osteoclasts by continued culture with 10ng/ml of MSCF and 50ng/ml of RANKL (Peprotech).

Co-culture. Raw264.7cells or primary bone marrow cells were plated on the bottom of a 6 well transwell plates in α-MEM with the insert containing either osteoblasts (MC3T3.E1#4) tumor cells (4T1 WT or 4T1ΔC) with and without EphA2 function respectively, or a combination of the osteoblasts with tumor cells. TRAP assays were performed to detect activated osteoclasts in vitro according to manufacturer's instructions

Results

EphA2 activity promotes breast cancer induced osteolysis

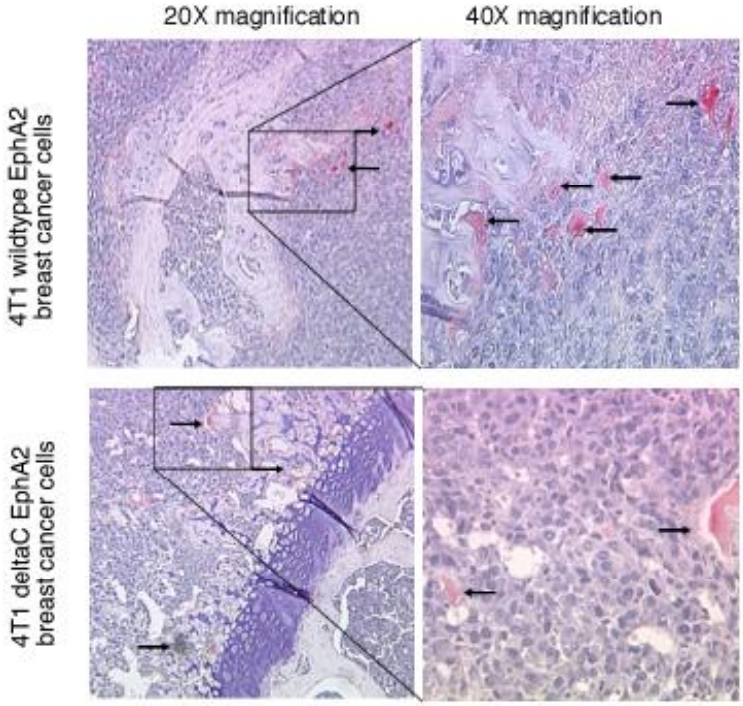
To determine if high levels of EphA2 could dysregulate bone remodeling and contribute to breast cancer bone, we expressed a cytoplasmic deletion mutant of

EphA2 (EphA2ΔC) in 4T1 mammary adenocarcinoma cells. The EphA2ΔC mutant was able to inhibit endogenous wildtype EphA2 signaling in a dominant negative fashion [11]. 4T1 breast cancer cells expressing either wildtype EphA2 or EphA2ΔC were injected into the tibia of recipient mice to determine the effect of EphA2 in breast cancer induced osteolysis. Balb/c mice injected with wildtype EphA2 4T1 cells demonstrated high levels of breast cancer induced osteolysis as observed by the absence of bone in microCT scans compared to those injected with 4T1ΔC (Figure 16A). Tibias collected from animals injected with these breast cancer cells were decalcified and processed for paraffin embedding and histomorphometry analysis. Tartrate resistant acid phosphatase (TRAP) combined with hematoxylin and eosin (H and E) staining revealed less bone in the parental injected samples in contrast to the 4T1ΔC though both demonstrated a high degree of tumor growth within the tibia (Figure 16B). Quantification of total bone versus total volume (BV/TV) revealed four times less bone in the wildtype EphA2 4T1 injected animals than the 4T1ΔC injected animals as a percentage of

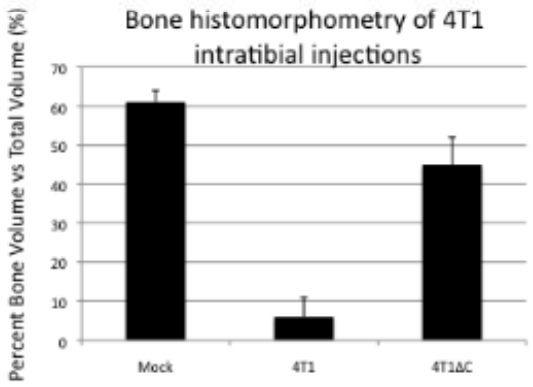
A


Wild-Type
4T1.ΔC

B


20X magnification
40X magnification
4T1 wildtype EphA2 breast cancer cells
4T1 deltaC EphA2 breast cancer cells

C


Bone histomorphometry of 4T1 intratibial injections
Percent Bone Volume vs Total Volume (%)
70
60
50
40
30
20
10
0
Mock
4T1
4T1ΔC

D

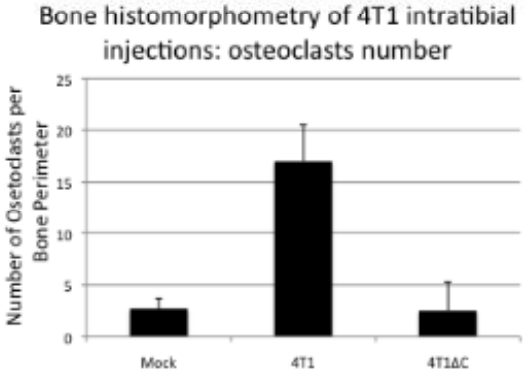

Bone histomorphometry of 4T1 intratibial injections: osteoclasts number
Number of Osetoclasts per Bone Perimeter
25
20
15
10
5
0
Mock
4T1
4T1ΔC

Figure 16. **EphA2 activity promotes breast cancer induced osteolysis in the intratibial bone metastasis bone model.** A) Live animal imaging reconstruction of CT scans demonstrating greater osteolysis in animals injected with wildtype EphA2 4T1 cells versus animals injected with 4T1ΔC cells. B) Representative TRAP staining of sections from animals injected with wildtype EphA2 or EphA2ΔC 4T1 tumor cells. Black arrows denote osteoclast resorbing bone. C) Quantifications of stained sections show a drastic decrease in bone volume versus total volume ratio (BV/TV) in the wildtype EphA2 4T1 cells relative to the 4T1ΔC cells (p=0.09). D) Quantifications of the number of osteoclasts in these sections also revealed a greater number of osteoclasts in the 4T1 wildtype EphA2 cells relative to 4T1ΔC (p=0.15).

bone remaining (Figure 16C). When paraffin embedded sections were stained for TRAP to detect osteoclasts, those animals injected with wildtype EphA2 4T1 cells had three times more osteoclasts than 4T1ΔC or needle punch controls (Figure 16C).

Since 4T1 is a murine mammary tumor line, we tested whether EphA2 also plays a critical role in human breast cancer cells. EphA2 activity was depleted in human MDA-MB-231 breast cancer cells, which express high endogenous EphA2 levels via a lentiviral delivered shRNA against EphA2. We confirmed stable knockdown by immunoblotting and TAQMAN qRT-PCR analysis (Figure 17 A and B respectively). Proliferative and apoptosis studies of EphA2 knockdown MDA-MB-231 cells revealed no significant differences in growth or death between knockdown and vector controls (Figure 17 C and D respectively). BrdU assays revealed a modest decrease in proliferation for tumor cells with decreased EphA2 expression similar to what was observed with the 4T1ΔC cell line [11]. TUNEL assays measuring apoptosis showed no differences in apoptosis *in vitro* upon loss of EphA2 also similar to what is recorded in the literature [11].

Utilizing the intratibial model for bone metastasis in mice, we investigated the role of EphA2 in mediating tumor cell-bone cell interactions in vivo in human breast cancer. We injected MDA-MB-231 vector control cells and MDA-MB-231 EphA2 KD cells via the tibia and monitored for development of tumors and lesions. Following approximately 6 weeks, the hindlimbs were collected and analyzed. With respect to tumor-induced osteolysis, the ratio of bone volume

(BV) versus total volume (TV) by microCt showed tumor-injected tibias with vector control MDA-MB-231 cells displayed more osteolysis than those injected with MDA-MB-231 EphA2 KD (Figure 18A). Bone histomorphometry on sections from these animals revealed greatly more osteoclasts in vector controls versus EphA2 KD cells as measured by TRAP staining. Moreover, vector controls cells also displayed reduced volume of bone relative to EphA2 KD cells (Figure 18B). Similar differences in bone volume (BV) versus total volume (TV) that were originally seen in the microCt analysis were also seen upon analysis of these bone sections. Furthermore, the faxitron images revealed larger areas of osteolysis in the vector control cells versus the MDA-MB-231 EphA2 KD cells (Figure 18C). These data suggest tumor cell EphA2 is able to promote breast cancer induced osteolysis in both mouse and human breast cancers.

EphA2 depletion inhibits tumor cell induced osteoclast differentiation

Numerous studies suggest tumor cell-bone cell interactions drive the vicious cycle that often induces osteolysis of the bone in breast cancer metastasis through increased activity of osteoclast function [222, 223]. Using a modified co-culture model we investigated the ability of tumor cell EphA2 to induce osteoclast differentiation with or without osteoblasts as measured by TRAP staining. In this assay primary bone marrow cells were plated underneath a transwell insert that was seeded with 4T1 tumor cells (wildtype EphA2 or EphA2ΔC) in the presence

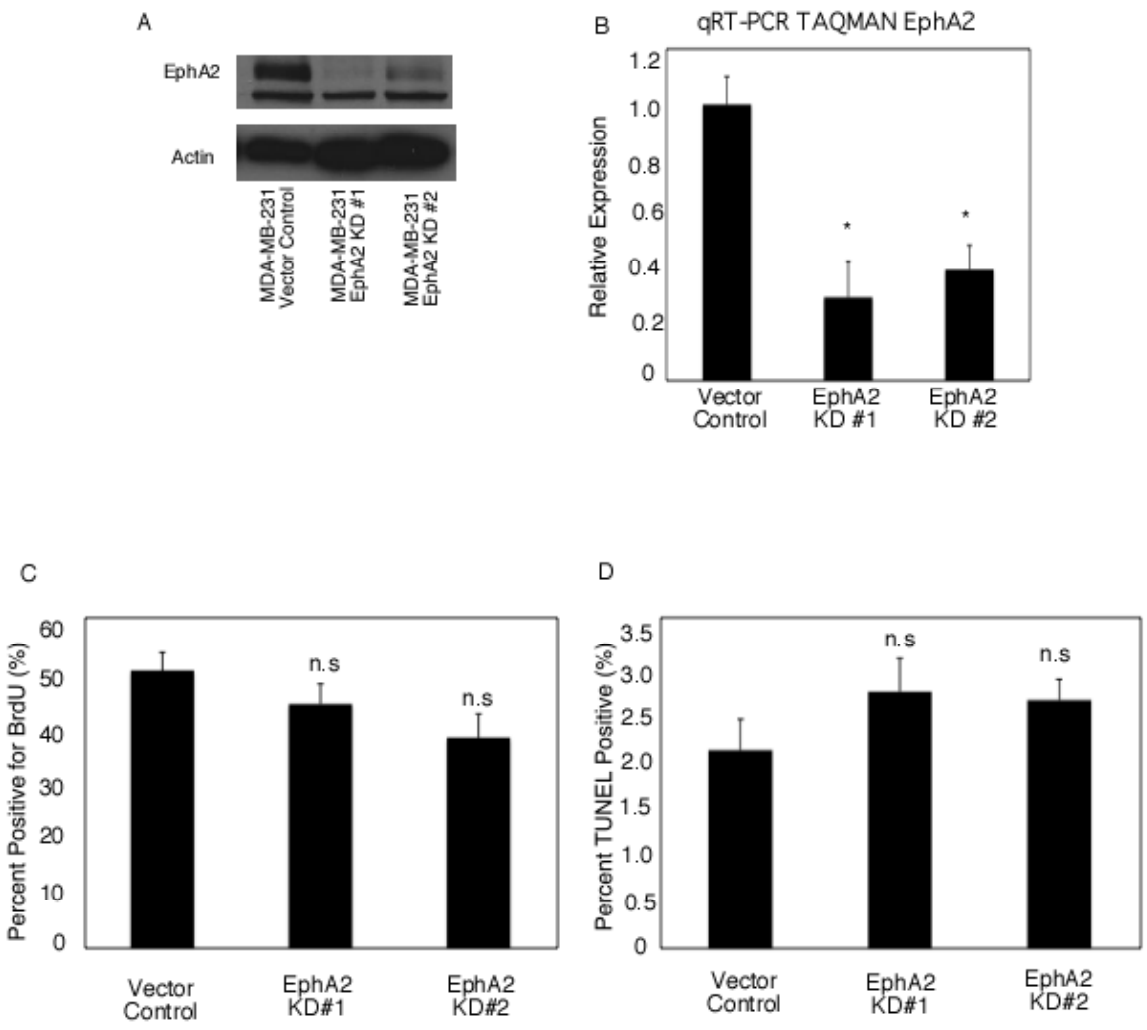


Figure 17. EphA2 knockdown in osteolytic breast cancer cells. A) Using lentiviral vectors, stable knockdowns for EphA2 were made in MDA-MB-231 breast cancer cells as shown by immunoblot. B) Further, analysis of knockdown of EphA2 was confirmed via realtime PCR using a Taqman assay for EphA2. Loss of EphA2 does not have a statistically significant effect on proliferation (C) or apoptosis *in vitro* (D). *p<0.05 n.s. not signifcant

or absence of osteoblasts to determine the ability of EphA2 to influence osteoclast differentiation (Figure 19A). Primary bone marrow cells cultured in the presence of tumor cells with ΔC expression had a reduced percentage of TRAP positive osteoclasts compared to wildtype EphA2 expressing cells (Figure 19B). Mature osteoblasts were also added to the tumor cells to see if physical interactions between the tumor cells and osteoblasts further induced the ability of osteoclast precursor cells to differentiate. We observed a minimal increase in percentage of TRAP positive osteoclasts when tumor cells and osteoblasts were cultured together versus tumor cells cultured without any osteoblasts. Though there was not a significant increase in percentage of TRAP positive osteoclasts when tumor cells were cultured with osteoblasts, the difference in percentage of osteoclast precursors that differentiated into TRAP positive osteoclasts was also reduced in the 4T1ΔC cells versus 4T1 wildtype EphA2 cells (Figure 19B). Human breast cancer cells plated in the aforementioned co-culture assay revealed a similar effect as the 4T1 murine breast cancer model. MDA-MB-231 vector control cells and MDA-MB-231 EphA2 KD cells seeded in the insert of a transwell filter with Raw267.4 cells, a mouse monocyte cell line, or primary mouse bone marrow cells underneath the insert enabled analysis of EphA2 on osteoclast differentiation (Figure 19C and D). Following five days of culture, cells plated in the lower chamber of the transwells were fixed and stained for TRAP to mark osteoclasts that differentiated from the precursor cells. EphA2 knockdown cells plated with RAW264.7 cells or with primary bone marrow cells significantly reduced the percentage of cells that differentiated into osteoclast relative to

vector controls (Figure 19C and D). These results suggest EphA2 is able to mediate osteoclast differentiation induced by breast cancer cells likely through regulation of soluble factors.

Osteoclast differentiation factors regulated by EphA2

Conditioned media collected from tumor cells in which EphA2 expression was diminished reduced the ability of osteoclast precursors to differentiate relative to vector control cells. Based on these data, we hypothesized that an EphA2 regulated soluble factor was responsible for tumor induced osteoclast differentiation. Using a RayBiotech mouse cytokine array we analyzed conditioned media from each condition in Figure 21 using the 4T1 wildtype EphA2 and 4T1ΔC cell lines. Several cytokines, chemokines, and growth factors displayed changes in expression level greater than two fold (Table 4). Many of these factors play important roles in bone biology including IL-6. Quantitative RT-PCR revealed decreases in IL-6 levels in 4T1ΔC cells. Similarly, MDA-MB-231 vector control cells had higher levels of IL-6 versus MDA-MB-231 EphA2 KD cells (Figure 22A). A human IL-6 ELISA assay was also performed on conditioned media collected from the MDA-MB-231 vector control cells and MDA-MB-231 EphA2 KD. We also observed a significant decrease in soluble IL-6 levels for MDA-MB-231 EphA2 KD cells versus MDA-MB-231 vector control cells (Figure 22B). Remarkably, further studies into important factors of bone

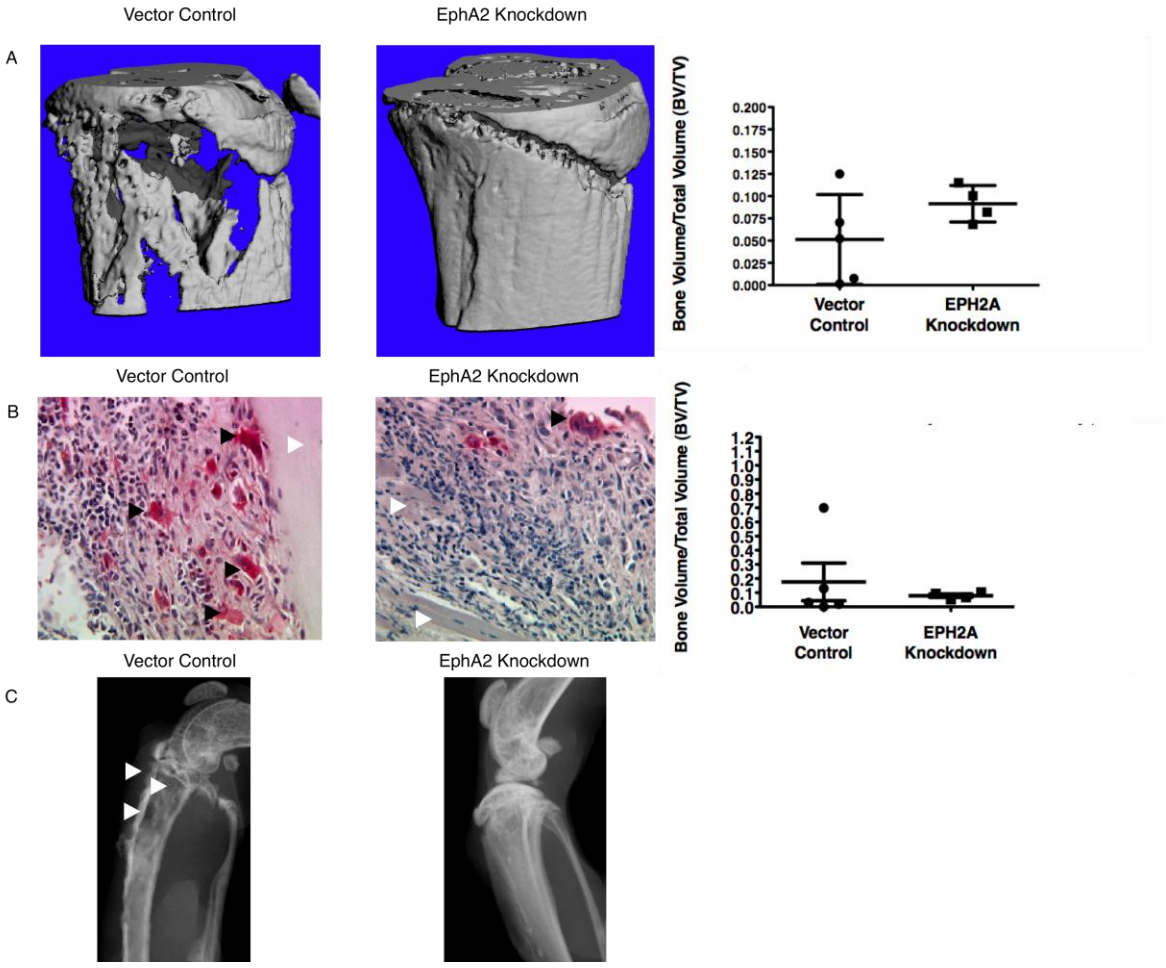


Figure 18. Tumor mediated osteolysis is attenuated by loss of EphA2. A) µCT scans of trabecular bone from animals injected with MDA-MB-231 tumor cells with EphA2 or knockdown cells of EphA2. BV/TV ratios were calculated and displayed to the right. B) Representative photomicrographs of histology from TRAP staining of EphA2 knockdown versus vector control cells displaying fewer osteoclast and more trabecular bone in the EphA2 knockdown tibia compared to the tibia injected with vector control breast cancer cells. White arrowheads indicate trabecular bone and black arrowheads indicate TRAP positive osteoclast. BV/TV quantification is displayed on the right. C) Faxitron imagining of EphA2 knockdown tumor cells and vector control tumor cells injected into animals. White arrowheads denote areas of osteolyssis and bone resorption seen more often in the vector control animals versus the EphA2 knockdown cells.

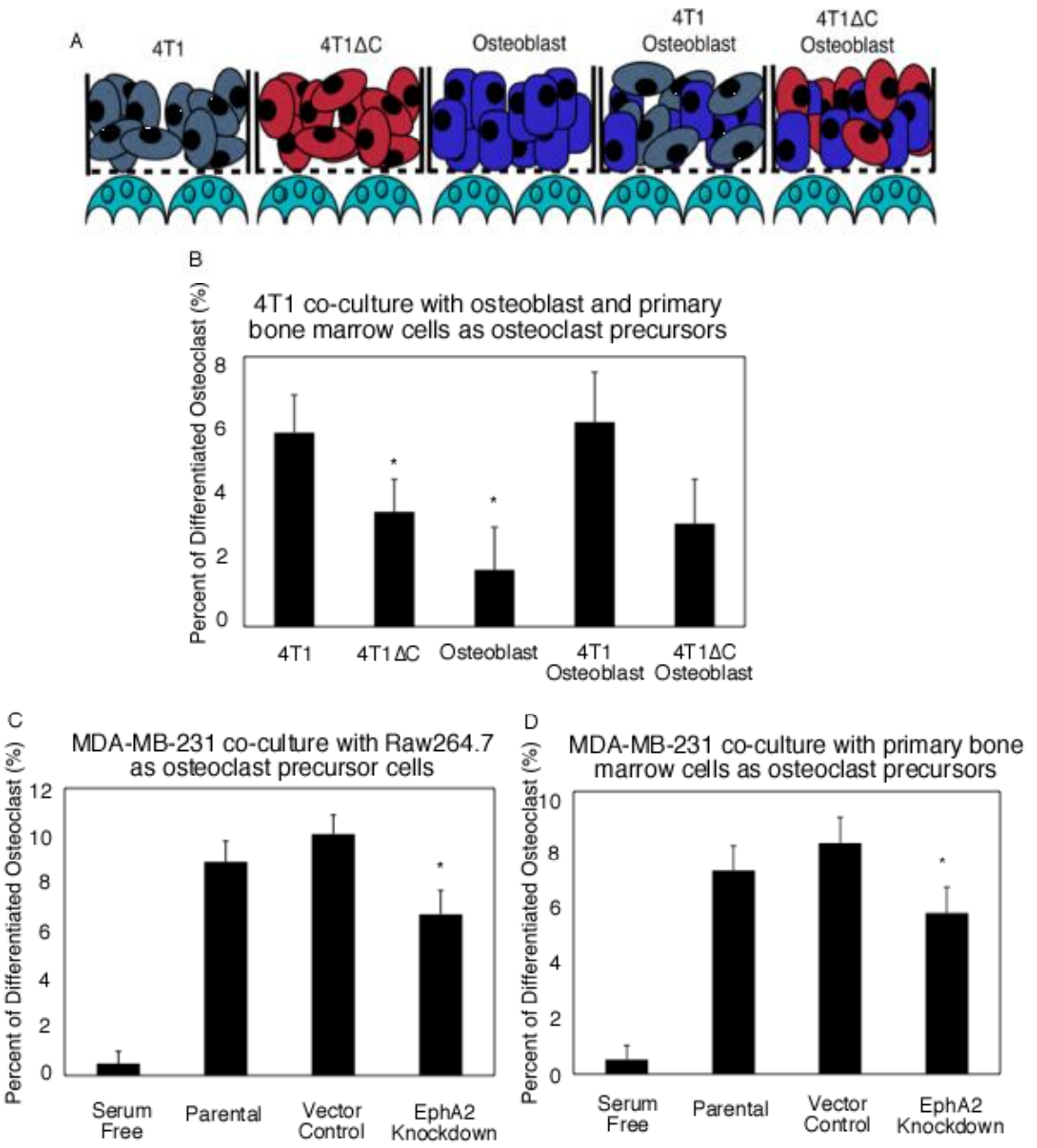


Figure 19. Loss of EphA2 activity inhibits differentiation of osteoclast precursors. A) Model for a modified co-culture assay used for analysis of tumor cell expressed EphA2 on differentiatin of osteoclast precursors in the presence or absence of mature osteoblasts. B) Percentage of differentiated osteoclast in 4T1 co-culture as measured by TRAP staining. C) Percentage of differentiated osteoclast from MDA-MB-231 co-culture with Raw 267.4 as osteoclast precursor cell following five days in culture. D) Quantification of differentiated osteoclasts from primary bone marrow cells following five days in culture after initial 3 day MCSF differentiation in MDA-Mb-231 co-culture. Data presented as percentage of total. *$p<0.05$.

Table 4. Mouse cytokine array targets. Array was incubated with conditioned media from 4T1 WT EphA2 or 4T1ΔC co-cultured with or without osteoblasts. Targets that had a 2 fold difference between WT EphA2 and ΔC expression are highlighted.

Axl	BLC	CD30	CD40	CXCL16	Eotaxin-2	FasL	IGFBP3	IGFBP5	Lix
L-Selectin	E-Selectin	MIP-1a	MIP-1g	PF4	P-Selectin	SDF-1a	TCA-3	sTNFRII	VCAM-1
GM-CSF	IFNg	IL-1a	IL-1b	IL2	IL-3	IL-4	IL-5	IL-6	IL-9
IL-10	IL-12	IL-13	IL-17	KC	MCP-1	M-CSF	RANTES	TNFa	VEGF
Eotaxin	MIG	G-CSF	GITR	ICAM-1	IGFBP2	IGFBP6	IGF-1	IL-12 p40/70	Leptin
MCP-5	MDC	MIP-2	MIP-3a	OPN	OPG	Resistin	SCF	TPO	VEGF-D

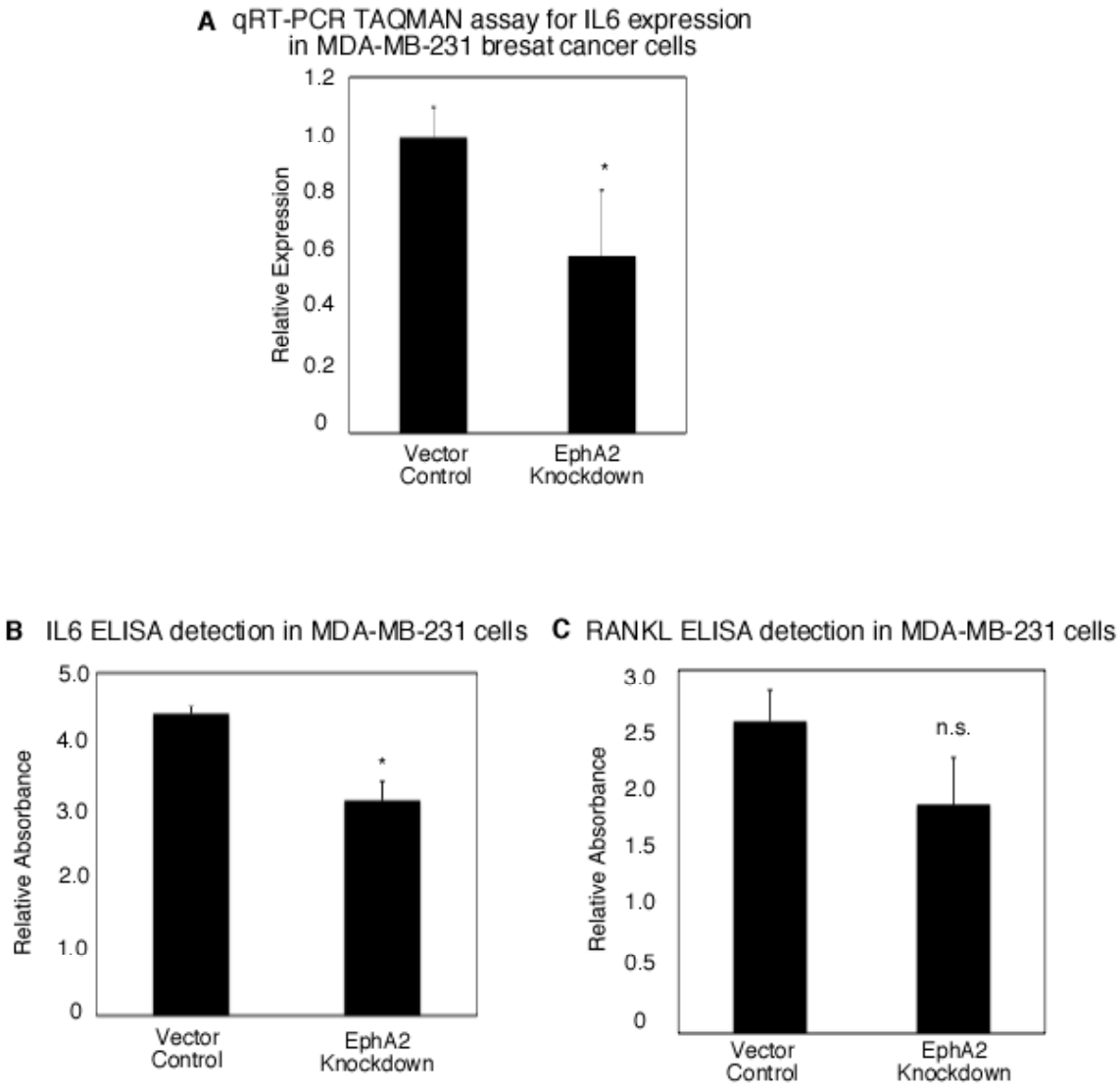


Figure 20. Effects of EphA2 expression on osteoclast differenction factors. A) Knockdown of EphA2 in MDA-MB-231 cells demonstrates reduced expression levels of IL-6 as measured by realtime PCR. B) Reduced expression of EphA2 in breast cancer cells leads to decreased release of soluble IL-6 but not RANKL (C) when using an ELISA for conditioned media detection. *$p<0.05$. n.s. not significant

remodeling i.e. RANKL and PTHrP, revealed no statistical changes. Reporter constructs developed in Dr. Greg Mundy's lab showed changes in EphA2 status of MDA-MB-231 cells as well as 4T1 cells did not result in changes to PTHrP activity (Julie Sterling and Greg Mundy personal communication). These results suggest a link between EphA2 expression and IL-6 expression, and that this tumor cell derived IL-6 could promote increased osteoclast differentiation.

Targeting EphA2 in breast cancer cells inhibits tumor induced osteolysis *in vivo*

Our studies thus far suggest that EphA2 enhances osteoclast differentiation. Thus, we hypothesized that blocking EphA2 activity could be a novel, effective new therapeutic approach to inhibiting breast cancer induced osteolysis. To investigate the efficacy of blocking EphA2 in breast cancer induced osteolysis we treated tumor-bearing animals with an activating antibody against EphA2 [191]. Following intratibial implantation of tumor cells, the anti-human EphA2 antibody or control human IgG was injected intraperitoneal every 72 until limbs were collected for analysis (Figure 21A). Seven weeks post implantation of MDA-MB-231 breast cancer cells animals were imaged for osteolytic response. Analysis of animals treated with the anti-EphA2 antibody revealed a decrease in osteolysis as compared to human IgG control when imaged with microCt (Figure 21B). With respect to tumor-induced osteolysis, analysis of the tumor injected tibias using TRAP staining and histomorphometry revealed significantly decreased osteolysis in the anti-EphA2 antibody treated animal versus the IgG control

treatment. The BV/TV ratio for the anti-EphA2 antibody treated animals were three times the total bone percentage of the IgG treated animals (Figure 21C). In addition, the number of osteoclasts per millimeter of bone perimeter was two times greater in the IgG control treated animals versus those receiving the EphA2 targeted treatment (Figure 21C). These findings suggest a role for EphA2 in breast cancer induced osteolysis that can be attenuated via blocking EphA2 receptor function.

Tumor cell EphA2 in close proximity to osteoclasts in human breast-to-bone metastasis.

Our animal models of tumor-bone interactions identified the connection between EphA2 activity and osteolysis. Furthermore, targeting human breast cancer bone xenografts with an antibody against EphA2 revealed the possibility of developing a more reliable inhibitor of breast cancer induced osteolysis. Based on this assessment, the expression of EphA2 was examined in human cases of breast-to-bone metastasis (n=4). Interestingly, EphA2 staining was seen throughout the tumor, but was more intense in areas closely associated with bone (Figure 22). More importantly, many of these areas stained for TRAP-positive multinucleated osteoclasts in the region that would be considered the tumor-bone interface. Thus, EphA2 may not only have a role in inducing

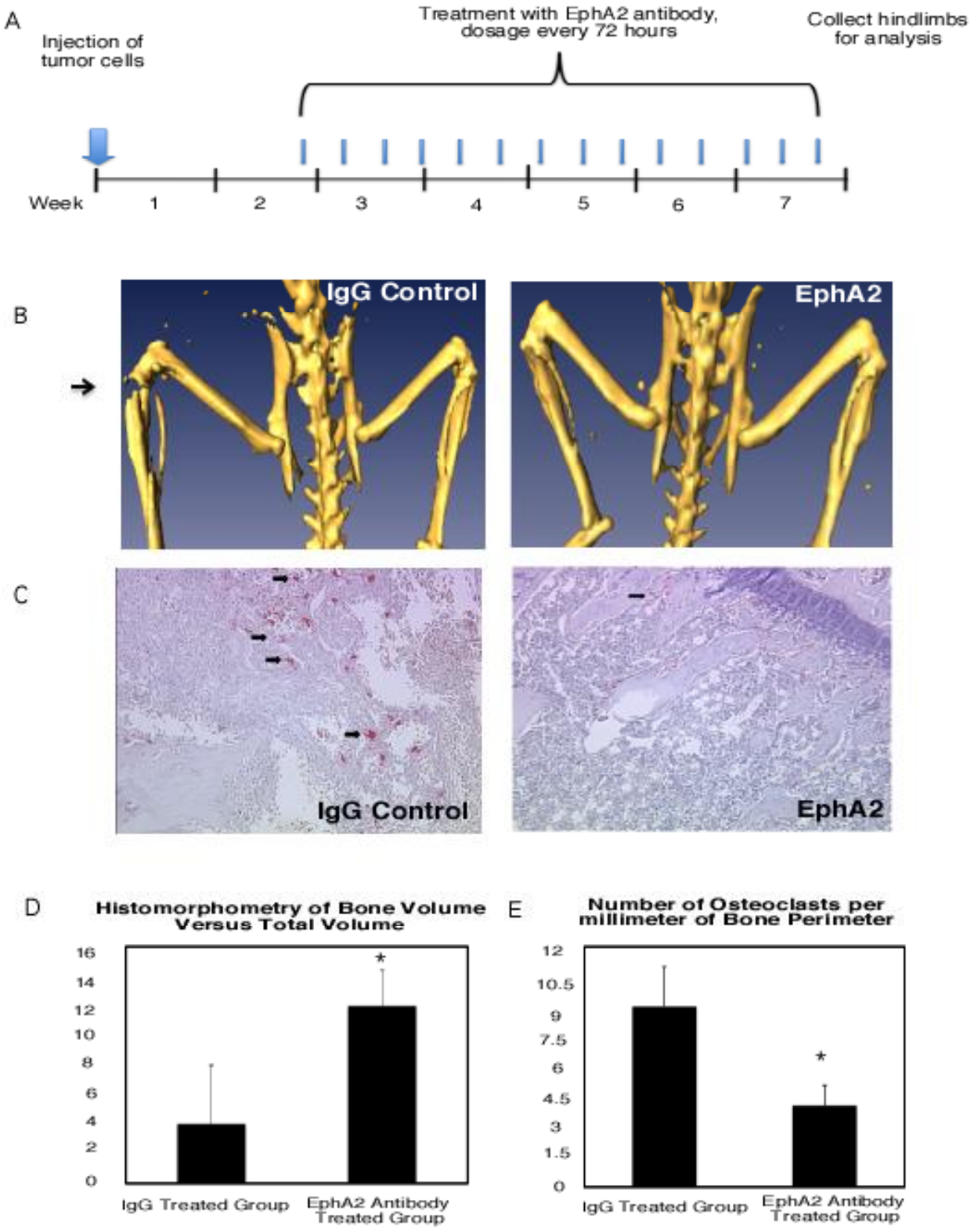

A
Injection of tumor cells
Treatment with EphA2 antibody, dosage every 72 hours
Collect hindlimbs for analysis
Week
1
2
3
4
5
6
7
B
IgG Control
EphA2
C
IgG Control
EphA2
D
Histomorphometry of Bone Volume Versus Total Volume
16
14
12
10
8
6
4
2
0
*
IgG Treated Group
EphA2 Antibody Treated Group
E
Number of Osteoclasts per millimeter of Bone Perimeter
12
10.5
9
7.5
6
4.5
3
1.5
0
*
IgG Treated Group
EphA2 Antibody Treated Group

Figure 21. A therapeutic antibody against EphA2 attenuates bone resorption. A) Treatment strategy for the application of the human antibody against EphA2 follwing MDA-MB-231 cell innoculation via intratibial injection. B) Live animal imaging reconstruction of CT scans demonstrating osteolysis in IgG control treated animals versus those treated with the EphA2 antibody. C) TRAP staining of sections from animals injected with tumor cells and treated with either IgG control or antibody against EphA2. Black arrows denote osteoclast resorbing bone. D) Quantifications of stained sections show a signifcant difference in bone volume versus total volume (BV/TV) in the EphA2 treated animals as well as showing a statistical decrease in number of osteoclast in the EphA2 treated animals versus controls. *$p<0.05$

soluble factors involved in osteoclast differentiation but may also have a direct physical interaction in mediating osteoclast differentiation and activity as suggested by localization of EphA2 tumor cells and osteoclast in human samples containing osteolysis.

Discussion

Accumulating evidence indicates that Eph receptors and associated ephrin ligands play critical roles in diverse cellular processes including cell growth and motility. More recently a role for these receptors and ligands in bone biology has emerged prompting the search for newly identified molecular pathways mediated by these unique receptor/ligand interactions in bone remodeling. Many of these molecular pathways focus on differentiation of osteoclasts and osteoblasts and their regulation of bone resorption or bone formation under homeostatic conditions [132, 143, 145]. However, high levels of Eph receptors are associated with various cancers that have the ability to metastasis to bone, including breast cancer [1, 74]. Lesions detected in bone from breast cancers are often osteolytic in nature, whereby activated osteoclasts cause bone resorption releasing growth factors that stimulate tumor cells to grow in the local environment in turn leading to the release of other growth factors by the tumors to induce continued bone resorption. Understanding the molecular mechanisms that control the vicious cycle is key to developing new therapies designed to treat bone metastasis and/or inhibit bone cell-tumor cell interactions that induce osteolysis.

Recent studies into Eph receptor function in cancers have shown regulation

of tumor growth by EphA2 via soluble EphA2-Fc receptor treatment as well as overexpression of dominant negative or kinase inactive forms of the receptor in 4T1 mammary adenocarcinoma cells [11, 88, 105]. Furthermore, studies using siRNA against EphA2 have demonstrated silencing EphA2 expression inhibits proliferation and tumor growth in mesothelioma and ovarian cancer [90, 91]. These reports reveal a direct role of EphA2 regulation in tumor growth and subsequent studies additionally revealed a significant role for EphA2 in promotion of tumor angiogenesis. EphA2 deficient studies revealed a failure of vascular endothelial cells to migrate, assemble, and incorporate into blood vessels when co-cultured with tumor cells *in vitro and in vivo* [88, 105]. In addition, tumor cells implanted into EphA2 null mice also show decreases in tumor volume, and microvascular density [105, 106]. Thus, an antibody against EphA2 would likely impact tumor growth and cytokine production (as demonstrated through EphA2 null studies of angiogenesis) simultaneously making EphA2 a great target for breast cancer metastasis.

Therefore, in the context of bone cell-tumor cell interactions, we hypothesize that blocking EphA2 will result in a decrease of osteoclast differentiation and activation thus breaking the vicious cycle and offering an effective means to control bone metastasis. This hypothesis is supported by our *in vivo* studies demonstrating the efficacy of a therapeutic anti-EphA2 antibody in reducing osteolytic disease in human breast cancer bone xenografts.

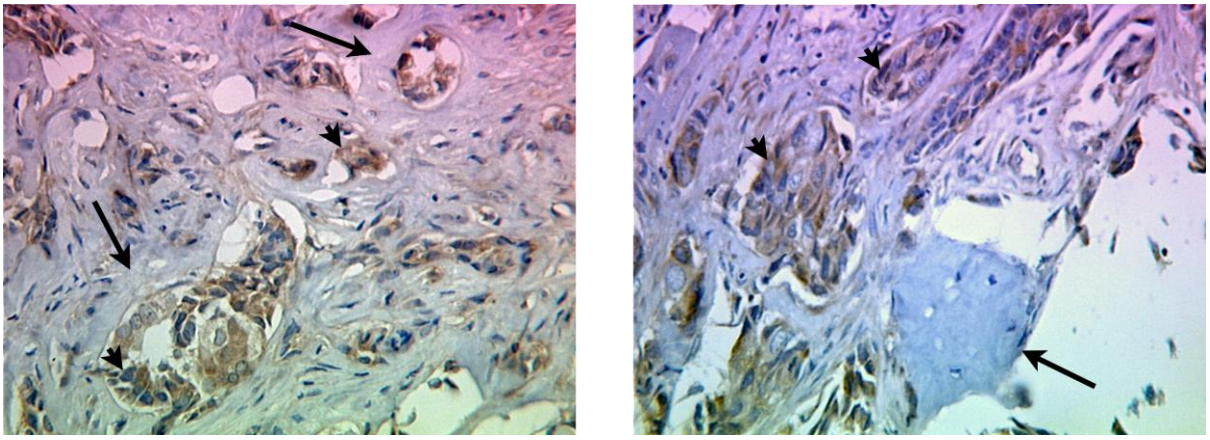


Figure 22. EphA2 localization to bone in human breast-to-bone metastasis. Tumor cell EphA2 positive staining was detected at the bone tumor interface and corresponded to areas of high osteolysis as measured by TRAP staining for presence of osteoclast. Representative staining of sections for TRAP and EphA2 are depicted in the above panels. Arrows denote bone and arrowheads denote EphA2 positive tumor cells at the bone tumor interface.

The first example of Eph/ephrin function in bone came using an ephrinB1 knockout mouse model that demonstrated skeletal abnormalities including cleft palate, skull shortening, asymmetric pairing of the ribs, and sternebral fusions [138]. Continued studies on class B Eph receptors and ligands also demonstrated an inhibitory function for ephrinB2 on osteoclast differentiation in contrast to the stimulatory function of EphB4 on osteoblasts [143]. Most recently, a similar function for A class Eph receptors in bone homeostasis was established. EphA2 on osteoblasts enhance osteoclastogenesis while inhibiting osteoblast differentiation through increased reverse signaling by ephrinA2 on osteoclast precursors [145]. In our studies of tumor cell induced osteolysis, we focused on tumor cell EphA2 forward signaling in regulation of soluble factors released from tumor cells to influence osteoclast differentiation *in vitro*. However, our *in vivo* studies, where physical interaction between bone and tumor cells is occurring, it is possible that a similar effect to bone homeostasis is being propagated through reverse ephrin signaling on osteoclasts. Increased expression of EphA2 on tumor cells could increase reverse signaling activity on osteoclast precursors leading to more osteoclast differentiation. Thus, tumor cells in this context mimic osteoblast function via binding to osteoclast precursors to promote more Eph/ephrin interactions and reverse signaling. This differentiation is inhibited when EphA2 expression is deleted via shRNA studies or with an active block of the EphA2 receptor i.e. activating antibody.

The temporal and spatial specificity of many of the signals transduced by Eph/ephrins ensures proper development of tissues and organs, but additional research suggests that aberrations in many of these same pathways also contributes to tumorigenesis and/or progression. Thus, it is likely these Eph and ephrin interactions have a key role in aberrant bone remodeling due to increased expression in tumors or bone cell-tumor cell interactions. Breast cancer and multiple myeloma are associated with bone metastases that exhibit high levels of osteolysis while prostate cancers usually have higher levels of bone formation, yet all have altered levels of Eph/ephrin signaling. In multiple myeloma, osteolysis is driven by lack of EphB4 expression on osteoblasts causing an inhibition of new bone formation (A. Bates, J Edwards personal communication). Tissue microarray data from prostate cancer metastasis foci in lymph node, liver, and bone revealed decreased ephrinA1 expression levels in bone metastasis [147], where-as other studies show decreased EphA1 receptor levels in osteolytic giant cell tumors of bone [148, 149]. The differences among these cancer models in bone response could be a result of differential regulation of Eph receptor and ephrin ligand expression and signaling. It is possible that reverse signaling by ephrin ligands promote osteoclast differentiation, and Eph receptor expression serves to promote or influence this reverse signaling. Studies demonstrate a decrease in EphB4 receptor levels are associated with an increase in osteolysis through the ability of ephrinB2 to transduce signals through its cytoplasmic domain that contains a phosphorylation site. This is in contrast to alterations in EphA class signaling where ligands do not have cytoplasmic

portions capable of phosphorylation. Thus, increased expression of EphA receptors can induce more reverse signaling of ephrin ligands where also triggering forward signaling of the receptor to release cytokines promoting osteoclast differentiation. The decrease in ephrinA1 levels of prostate cancer metastasis to bone and the increase in bone formation of this model is most likely due to decreased osteoclast activity. The loss of ephrinA1 on the osteoclast precursor's surface attenuates and limits the response of EphA receptors that would bind to induce reverse signaling and osteoclast differentiation. Thus, coupling is disrupted and an imbalance favoring bone formation results.

Inhibiting EphA2 by deletion or blocking activity leads to impaired osteoclast development as measured by osteoclast differentiation assays and functional assays including in vivo analysis. Our studies have also revealed decreases in cytokine production by cultured tumor cells (both human and murine) when EphA2 activity is inhibited. The list of cytokines decreased upon EphA2 inhibition consists of many inflammatory factors that have been associated with osteoclast precursors and mature osteoclast function (e.g. GMCSF, MCSF, MIP1, and IL-6). IL-6 has the capacity to influence many biological events including bone remodeling through stimulating production of PTHrP that decreases production of the RANKL decoy receptor OPG (osteoprotegerin) and increases the osteoblast production of RANKL. In our studies changes to OPG and PTHrP were not detected and RANKL was not changed upon inhibition of EphA2. It has been well recognized, particularly in multiple myeloma, that the interaction between stromal cells and tumor cells in the bone marrow contributes to tumor progression [224].

Adhesion of myeloma cells to MSC (mesenchymal stem cells) through cell adhesion molecules or integrins induces the expression of IL-6 by MSC [225]. We first suspected this was the case as well, but our studies have revealed that tumor cells are able to release these cytokines independently of MSC or osteoblasts suggesting that cell-cell contact is not required. Thus, tumor cell expressed EphA2 controls the release of soluble IL-6 into condition media. How EphA2 regulates IL-6 release and potential function upon osteoclast precursors is not well understood, though we are currently investigating this.

In conclusion this study reveals a novel function of EphA2 signaling in tumor cell-bone cell interactions involved in osteoclastogenesis and osteolysis associated with breast cancer metastasis. Furthermore, our data suggest the benefits for targeting EphA2 in advanced stage breast cancer disease associated with bone metastasis to disrupt the vicious cycle. Our data support the rationale for continued investigation into targeting cell-cell interactions via Eph receptors and ligands as a way to treat osteolytic bone disease that would offer the potential for increased therapeutic options available to patients suffering from this painful disease.

CHAPTER IV

CONCLUSIONS

The Eph receptors and their corresponding ephrin ligands are a relatively young receptor tyrosine kinase family. The first member of this family was discovered a little over 20 years ago, but since then the study of this, the largest known family of receptor tyrosine kinases, has evolved considerably. Over the past 20 years, the known biological functions of Eph receptors and their ligands have experienced tremendous growth, as has the scope and diversity of Eph and ephrin-mediated signaling. Correspondingly, scientific interest in the field has grown during this time as measured by the boom in publications tallied through Pubmed. Eph receptors and ephrins have emerged as key regulators of physiological and pathological processes in multiple diseases and during normal homeostasis and development. Despite our current knowledge of Eph receptors and their ligands in biology, a more complete understanding of function and dysregulation in the context of cancer as well as development has yet to be achieved. The work presented here focuses on one specific Eph receptor and represents the balance between developmental biology and metastatic cancer through the function of this receptor, EphA2, and its ligands.

In this thesis we demonstrate for the first time the developmental effect of EphA2 receptor regulation of normal growth and branching morphogenesis in the mammary gland. Mammary epithelial branching morphogenesis is a complex developmental process during which an extensive network of branched ducts forms from a rudimentary epithelial bud [reviewed in [64, 201]]. In response to hormonal stimuli, terminal end buds (TEB) form at the tips of the ducts and invade into the surrounding stroma. New primary ducts then form by bifurcation of the TEBs and secondary side-branches sprout laterally from the trailing ducts. This process is reiterated through branching and tissue remodeling until the entire mammary fat pad is filled with a ductal tree in the virgin gland.

Wildtype animals at the onset of puberty, approximately 3.5 weeks, will undergo rapid proliferation and invade the stroma directionally from the rudimentary mammary epithelium anlagen, giving rise to a fully branched ductal tree that completely fills the mammary fat pad by 10 weeks of age. EphA2 deficient animals, in contrast, exhibit severe growth retardation displayed by reduced fat pad filling and penetration through the mammary fat pad as measured by whole mount analysis (Chapter II). This defect is more prominent at 5 and 6 weeks of age and can persist through adulthood despite some compensation taking place [5]. The defects in outgrowth and branching in EphA2 deficient mice are a result of reduced proliferation as determined by tissue staining for PCNA, and not related to apoptosis as measured by TUNEL. High proliferative activity was observed in 5 and 6 week old animals, while EphA2 deficient animals had reduced PCNA staining in age matched animals. The

differences in proliferation paralleled the defects observed in the whole mount analysis of age group involved in the study. Thus, loss of EphA2 impairs normal development and architecture of the mammary epithelial tree.

Mammary gland branching morphogenesis is regulated by endocrine hormones and local paracrine interaction between the developing epithelial ducts and their adjacent mesenchymal stroma. Based on reciprocal transplantation studies we were able to show EphA2 function was specific to mammary epithelium, rather than mesenchymal stroma, in regulation of growth and branching of the mammary gland ducts. Although the mediators of the complex interaction in mammary gland development are not fully characterized, receptor tyrosine kinases (RTK) are among the critical regulators of branching morphogenesis [57]. Hepatocyte growth factor/scatter factor (HGF/SF), a mesenchymal derived mitogen and morphogen, induces branching morphogenesis through its receptor c-Met, which is expressed on mammary epithelial cells [reviewed in [202, 203]]. A number of factors acting in a paracrine fashion are known to regulate mammary gland development via branching morphogenesis [212]. In particular, HGF promotes ductal outgrowth and tubule formation in the mammary gland [202].

Data derived from reciprocal transplant experiment were validated in an in vitro system where specific morphogens can be manipulated to determine their effect on mammary branching. Primary mammary cells isolated from wild-type animals branched and invaded the three-dimensional matrix where-as cells isolated from the EphA2 null animals displayed diminished branching relative to

the wild-type controls. In response to HGF stimulation, wild-type spheroids undergo extensive remodeling and branching. In contrast, EphA2-deficient spheroids fail to undergo branching morphogenesis, displaying significantly fewer branches relative to wild-type cells in response to HGF stimulation. Overexpression of wild-type EphA2 receptor in primary mammary epithelial cells via adenovirus transduction not only rescued phenotypes in EphA2-deficient cells, but also drastically enhanced branching morphogenesis in wild-type cells. Thus, we have shown EphA2-deficiency inhibits HGF-induced mammary epithelial cell branching morphogenesis.

Dynamic regulation of the actin cytoskeleton is critical in a number of cellular processes including cell migration and branching morphogenesis. RhoA GTPases are key regulators of actin stress fiber formation and are necessary for cell migration [213, 214]. Moreover, Ewald et al. recently reported that inhibition of ROCK kinase, a downstream effector of RhoA, results in hyperbranched mammary epithelium in organoid cultures [216], suggesting that RhoA is crucial for proper branching morphogenesis in mammary epithelial development. We show here through inhibitor studies that EphA2 functions downstream of HGF to regulate RhoA GTP to induce mammary gland branching. Furthermore, we show that a balance of RhoA is critical as too much or too little RhoA leads to similar observations.

The data reported herein also shows for the first time the function of EphA2 in breast cancer induced osteolysis. Though other studies have demonstrated the effects of Eph receptors and their ligands in maintaining bone homeostasis we

show the effects of EphA2 in the context of tumor progression. Our studies demonstrate the ability of breast tumor cells with high levels of EphA2 to induce osteolysis in the bone through increased osteoclast differentiation that eventually leads to more functionally active osteoclast (Chapter III). Inhibition of EphA2 activity in human and mouse mammary adenocarcinoma cells leads to decreased osteolysis *in vivo*, whereas *in vitro* we see inhibition of osteoclast differentiation as measured by TRAP staining. More importantly our studies show the efficacy of a therapeutic antibody targeting EphA2 *in vivo* that leads to reduced osteolysis as measured by microCt scanning and bone histomorphometry analysis. The efficacy of this antibody demonstrated here suggests the potential benefit for use in inhibition of bone osteolysis.

Future Directions

The ability of Eph receptors to mediate ligand independent and dependent signaling that promote different outcomes is one of the main reasons why further studies should be conducted on Eph/ephrin signaling. Furthermore, the controversy that exists in the field of Eph/ephrin biology in terms of oncogenic versus tumor suppressive functions and the context dependent signaling displays the many questions that remain to be answered [226]. One specific question that must be addressed involves teasing out specific signaling activities of different Eph receptors in response to or in the absence of their ligands. Our lab has developed a working model to conceptionalize the paradoxical effects of Eph signaling (Figure 23). Under physiological conditions, Eph receptors and ligands

interact at the cell surface, inhibiting activation of various pathways through ligand-mediated receptor internalization and degradation. Blockade of these pathways by Eph receptors and their ligands is critical in development as demonstrated by studies showing aberrant neuronal circuitry, remodeling of blood vessels, tissue homeostasis, tissue boundary formation, and glandular development when Eph/ephrin interactions are disrupted (Chapter II and reviewed in [1, 227]. We believe cancer cells, however, have developed mechanisms to prevent ligand dependent signaling leading to tumorigenesis. Disruption of cell-cell junctions and mislocalization of Eph receptors and ephrins on the cell surface is one such mechanism. Also, studies have demonstrated the ability of known oncogene products in cancers to cross talk with Eph receptor kinases through heterodimerization in order to cause activation of downstream cascades independent of any ligand binding (Table 1 and references therein). Therefore we hypothesize ligand dependent signaling apparently promotes tumor suppressive functions, where-as ligand independent signaling likely induces an oncogenic function.

Future studies using single and double knockout mouse models to delineate the differences between receptor and ligand function in both development and

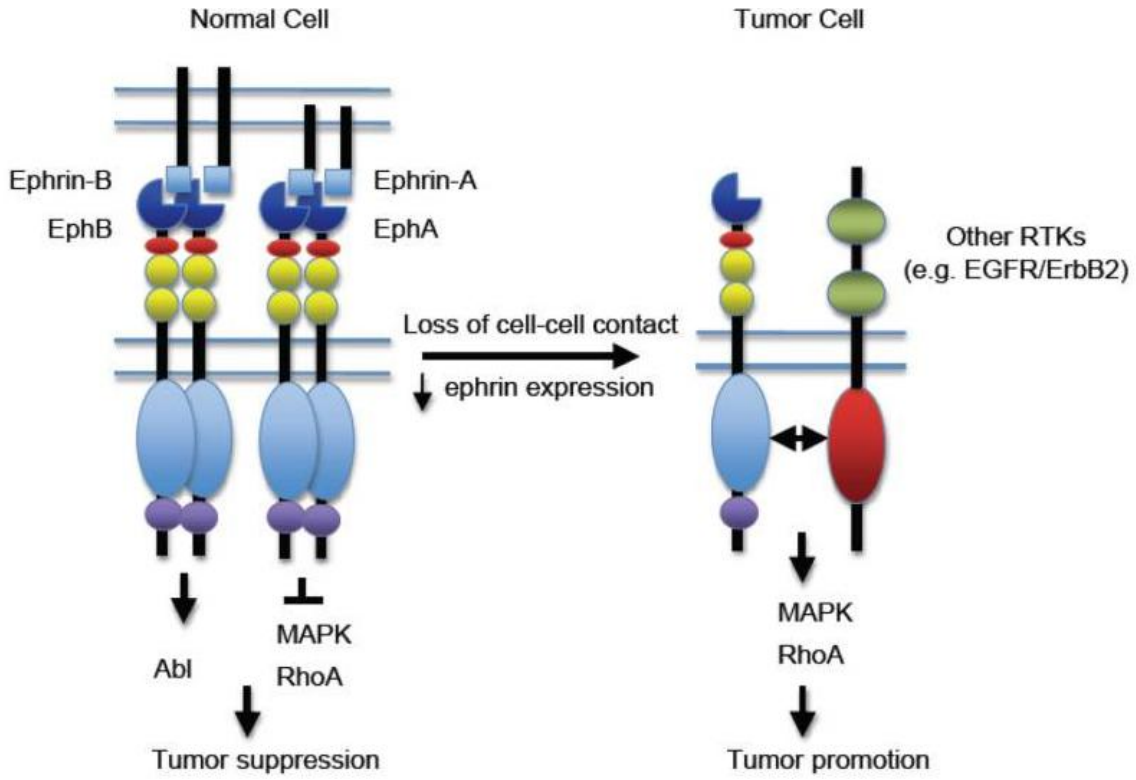


Figure 23. **Working model for Eph receptor function in tumor promotion and tumor suppression**. In normal cells, engagement of Eph receptors with ephrins on adjacent cells *in trans* induces receptor forward signaling, leading to inhibition of Ras/mitogen-activated protein kinase (MAPK) activity, or suppression of Crk activation via Abl kinase activity, and tumor suppression. In tumor cells, disruption of cell-cell junctions inhibits Eph receptor interaction with endogenous ephrins *in trans*. In addition, Eph receptors are often upregulated whereas ephrins are downregulated. Crosstalk between Eph receptors and other receptor tyrosine kinases such as ErbB2 and epidermal growth factor receptor (EGFR) results in increased activity of the Ras-MAPK pathway and the RhoA GTPase, and enhanced tumor malignancy. From [167]

tumorigenesis would be very beneficial in confirming this hypothesis. To complement the studies done with the EphA2 KO animal in development (see Chapter II) and tumorigenesis [5] we have proposed and begun studies to characterize the ephrinA1 KO animal in development and tumorigenesis. The EphA2 KO and ephrinA1 KO animals are ideal tools to begin a study to sort out receptor/ligand signaling complexes in context dependent situations. Independent investigations and characterization of the ephrinA1 KO mouse will follow a similar approach as outlined in Chapter II for the EphA2 KO mouse to determine the function of ephrinA1 on mammary gland development and tumorigenesis. As discussed already, in normal cells EphA and ephrinA are expressed at low levels and interact in trans between adjacent cells [5, 35, 88]. In addition my work on EphA2 deficiency suggest an impaired mammary epithelial growth and branching while work from our lab reported impaired ErbB2 dependent tumorigenesis *in vivo* [5, 69]. Preliminary data of the ephrinA1 ligand knockout mouse, in contrast, shows increased epithelial branching and hyperplasia. Analysis of EphA2 and ephrinA1 in a panel of human breast cancer cell lines revealed that EphA2 and ephrinA1 expression are mutually exclusive and this expression pattern is seen in a significant portion of lymph node metastasis from human breast cancer samples [8]. Thus, we hypothesize an interaction between EphA2 and ephrinA1 inhibits growth and invasiveness in the mammary gland. Our theory suggest ephrinA1 KO animals would suppress EphA2 forward signaling and receptor internalization and degradation thereby leading to inhibition of quiescent epithelial cells. This disruption of EphA2 and

ephrinA1 signaling would enable EphA2 protein levels to increase on the membrane surface to promote growth through the Ras/Erk and Rho pathways leading to malignant transformation and further elevating the oncogenic effect of EphA2.

Eph receptor crosstalk, ligand independent signaling

Another approach to understanding the roles of ligand-dependent and ligand-independent signaling of EphA2 will be to take the advantage of the crosstalk between EphA2 and ErbB2. Reports from our lab demonstrated a physical interaction between EphA2 and ErbB2 as well as a functional interaction leading to phosphorylation of EphA2. In addition, this crosstalk leads to therapeutic resistance in breast cancer and speculation of such in lung cancer. Crossing the ephrinA1 KO animals with the MMTV-Neu breast cancer model should assist in determining the ligand dependent and independent signaling roles in malignancy. I would expect that ephrinA1 KO animals would have increased branching and growth in development of the epithelial (opposite phenotype of EphA2 receptor KO) in the context of mammary gland development. Also, we would expect these animals to display mammary epithelial hyperplasia and hyperbranching and potentially develop tumors spontaneously. Outside of development, we hypothesize the ephrinA1 KO animals crossed to the MMTV-Neu breast cancer model will enhance tumorigenesis as well as metastasis due to the suspected release of inhibition on EphA2. It would be interesting to see if the deficiency of ephrinA1 can disrupt interactions with EphA2 to accelerate tumorigenesis in this

model since the native ligand is gone. I would expect a reduction in tumor latency and an increase in tumor volume and propensity to metastasize when ephrinA1 is absent from the cell surface allowing EphA2 receptors to remain on the cell surface and not be internalized.

Furthermore, utilizing the ephrinA1 KO animal and mating it to the EphA2 KO animal for a double KO could be used to determine if tumorigenesis enhanced by ephrinA1 deficiency is EphA2 dependent. Loss of both the receptor and ligand likely will reduce tumorigenesis to mimic the latency and progression in the parental MMTV-Neu model. Likewise, the double knockout could mimic a wild type control animal in terms of developmental despite the function of Ephs and ephrins in heart development, vessel formation, tissue boundaries, and neuronal patterning due to the loss of the both receptor and ligand leading to a balance as opposed to an imbalance with single knockouts. Investigations into the role of EphA2 as an oncogene could be greatly enhanced as well with the assistance of the ephrinA1 KO model. An EphA2 mammary epithelial transgenic mouse model has been developed in our lab and preliminary results show overexpression of EphA2 increases tumor onset, tumor burden, tumor number and metastasis. However, these values are modest and confirming overexpression by immunoblot has been difficult. We believe that this modest response, despite having a homozygous line for the EphA2 transgene, could be a result of endogenous ephrinA1 expression limiting the degree by which EphA2 is overexpressed on the cell surface. Thus a critical experiment in determining the oncogenic effect of EphA2 would be through crossing this transgenic animal for

EphA2 with the ephrinA1 KO to potentially increase and stabilize the expression levels of EphA2. I would expect a much greater expression of EphA2 and subsequent increases of tumor onset, burden, numbers and metastasis beyond modest differences.

Reverse signaling via ephrin ligand

One of the unique characteristics of Eph/ephrin signaling is the ability of the receptor expressing cell as well as the ephrin expressing cell to function in signal transduction pathways. The ability of ephrin ligands to signal is known as reverse signaling and is an area of active investigation with the A class ephrins. Using ephrinA1 KO animals alone or crossed with breast tumor models we will have the opportunity to investigate the role of A class reverse signaling in cancer cells as the context of signaling has proven to be critical in response or outcome. Most of the studies that have been presented thus far have focused on the Eph receptor and understanding how it signals in development, tumor development, and interactions with the microenvironment. Ephrin ligands are also present on tumor cells suggesting they may also have a role in tumorigenicity via reverse signaling. Similar to the Eph receptors, both pro- and anti-tumorigenic properties have been attributed to ephrin molecules themselves. EphrinB1 tyrosine phosphorylation disrupts binding of Par6, a scaffolding protein, to promote tight junctions and anti-tumorigenic potential in colon cancer cell studies [228]. EphrinA5 also demonstrates anti-tumorigenic properties in glioma through down regulation of EGFR levels [229]. In contrast, ephrinA5 reverse signaling

activates Fyn to induce murine fibroblast transformation measured by cell growth in soft agar, invasion, and morphology changes [230]. Reports for ephrinB reverse signaling suggest lipid raft localization induces Rac1 activation to increase cellular migration, invasion and pro-tumorigenic activities [231-233]. Continued research with transgenic and knockout mouse models much like those proposed earlier in this section will be instrumental in determining the precise roles of reverse signaling.

Role of EphA2 in bone development and osteolysis

Other important studies center on the role of ephrin ligand and Eph receptors in bone homeostasis as well as tumor induced osteolysis. Considering that ephrin ligand and Eph receptor interactions are critical in bone homeostasis for both the A class and B class receptors [143, 145], it will be interesting to look at bone development in both the EphA2 KO and ephrinA1 KO animals to see if there are differences in bone density as measured by microCt or faxitron analysis. We would expect there to be some sort of difference in bone density since the ability of osteoclast and osteoblast cells to directly interact would be inhibited with the loss of the receptor or ligand respectively, thus causing a block in bidirectional signaling.

Our study has focused and investigated the final stage in the metastatic cascade by looking at tumor cell bone cell interactions. Previous results from our lab have demonstrated the loss of EphA2 can reduce the ability and degree by which breast cancer cells metastasize [11]. It would be interesting to see how

orthotopic injections of tumor cells lacking EphA2 function would affect bone metastasis, and whether EphA2 is only involved in osteoclast differentiation and function or if there is a greater role for EphA2 in directing cells to the bone. Likewise, intratibial injections into the ephrinA1 KO mouse model would be of great interest to see if host loss of the ligand exacerbates the ability of EphA2 to induce osteolysis.

Targeting EphA2 as a treatment

Currently there is no cure or effective treatment available for patients suffering with bone metastasis. The treatments available for these patients such as bisphosphonates, radiation, surgery and chemotherapy are only palliative. Finding new mechanisms underlying cell-cell communication between bone cells and tumor cells is key for developing better more effective treatments against bone metastasis. Our results suggest that targeting of Eph receptors, specifically EphA2, would be beneficial to patients through inhibition of osteoclast differentiation and subsequent activation. The strength of EphA2 as a drug target lies in the expression profile of the protein. It is found in many adult epithelial tissues such as brain, skin, ovary, and breast but at low levels [234]. Cancers arising from these epithelial tissues, conversely, are usually associated with high expression levels of EphA2 [1, 74]. The abundance of EphA2 on tumors provides an ideal cancer target as the EphA2 antibody can target the more abundant tumor EphA2 opposed to less expressed normal tissue EphA2 receptor levels. The lower expression of EphA2 in normal tissue also will limit

the toxicity and side effects associated with this treatment, as the antibody would target the more abundant tumor EphA2. Phase I trials looking for adverse effects associated with treatment would need to focus on tissues composed of high levels of epithelial cells (i.e. kidney, skin, intestines etc.) where EphA2 would be expressed normally to investigate the ability of targeting EphA2 and binding on normal tissue. In addition, wound healing could potentially develop as an adverse effect with studies showing a critical role between EphA2 receptor and ephrinA1 ligand in angiogenesis (reviewed in [206, 235]).

Patients selected for inclusion in a trial investigating the toxicity and efficacy of an EphA2 targeting antibody would have positive staining for EphA2 in their primary breast tumor by IHC as a vast majority of breast cancers have increased EphA2 expression but not all do. Furthermore, preclinical data presented in this thesis would suggest a better response in patients that have advance breast cancer with metastasis to bone. Using the aforementioned selected patient cohort, the primary outcome measure of the clinical trial would focus on the response in osteolytic lesions associated with breast cancer metastases to bone. Inhibition of new osteolytic lesions or reductions in lesion number or size from decreased osteoclast function would be viewed as a positive sign for EphA2 targeted treatments in breast cancer bone metastasis. Continued studies and a larger multi-centered trial would assist in determining if an anti-EphA2 antibody treatment alone or as part of a combination offered a better overall response, longer duration, and higher progression free response versus the current chemotherapy standard of care in breast cancer metastasis to bone

bisphosphonates.

Aside from attenuation of osteolysis in breast cancer metastasis to bone via inhibition of EphA2, it would be beneficial to see if cell-cell interactions between Eph receptors and ephrin ligands are critical in the ability of the primary tumor to metastasize to bone. An important study would be to use the knockout mouse models developed in our lab for the Eph receptors and ephrin ligands and combine them with orthotopic models of cancer. This study has been proposed and attempted in our lab, however the majority of cells colonize the lungs before they colonize the bone as they metastasize. Thus, results in these studies are often inconclusive due to the animal succumbing to metastatic disease in the lungs before bone lesions are detectable. Intracardiac injections, as well as the intratibial injections, are feasible methods used to circumvent the lung metastases, however these are not effective alternatives to looking at cell-cell interactions through the metastatic process that occur in orthotopic xenografts. The development of particular breast cancer cells with a higher propensity to metastasize to bone rather than lung are under development by multiple groups for future use in understanding metastasis [236-238]. These studies would help in determining a potential role of Eph receptors in the “bone metastasis niche,” likewise utilizing the ephrinA1 KO animals we can investigate the potential contributions from host derived Eph/ephrin interactions in metastasis. Considering the role of ephrinA1 and EphA2 in tumor angiogenesis it is likely that tumor-host interactions have a critical role in metastasis leading up to tumor cell bone interactions.

In conclusion we have shown a role for EphA2 in mammary gland development through regulation of Rho activity and epithelial branching influenced by HGF. In addition, this dissertation highlights the detrimental effects of EphA2 expression on breast cancer cells that are able to interact with existing stroma of the bone to induce osteoclast differentiation and activation that leads to osteolysis. Therefore, novel therapeutic agents focusing on major components of the vicious cycle and/or blocking cell-cell interactions between bone and tumor cells will improve the current treatment options offered to patients suffering from lytic bone disease. Continued development of specific inhibitors to Eph receptors or therapies that block their activity, like those highlighted and included in this dissertation, would benefit many of these patients who have a poor response to bisphosphonates or other traditional treatment methods. Furthermore, targeting Eph receptors and their ability to mediate activity via cell surface binding may be helpful as adjuvants in combination with bisphosphonates or other inhibitors.

REFERENCES

1. Pasquale, E.B., *Eph-ephrin bidirectional signaling in physiology and disease.* Cell, 2008. **133**(1): p. 38-52.

2. Kullander, K. and R. Klein, *Mechanisms and functions of Eph and ephrin signalling.* Nat Rev Mol Cell Biol, 2002. **3**(7): p. 475-86.

3. Fang, W.B., et al., *Identification and functional analysis of phosphorylated tyrosine residues within EphA2 receptor tyrosine kinase.* J Biol Chem, 2008. **283**(23): p. 16017-26.

4. Binns, K.L., et al., *Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors.* Mol Cell Biol, 2000. **20**(13): p. 4791-805.

5. Brantley-Sieders, D.M., et al., *The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling.* J Clin Invest, 2008. **118**(1): p. 64-78.

6. Pratt, T., et al., *The winged helix transcription factor Foxg1 facilitates retinal ganglion cell axon crossing of the ventral midline in the mouse.* Development, 2004. **131**(15): p. 3773-84.

7. Pratt, R.L. and M.S. Kinch, *Ligand binding up-regulates EphA2 messenger RNA through the mitogen-activated protein/extracellular signal-regulated kinase pathway.* Mol Cancer Res, 2003. **1**(14): p. 1070-6.

8. Macrae, M., et al., *A conditional feedback loop regulates Ras activity through EphA2.* Cancer Cell, 2005. **8**(2): p. 111-8.

9. Noren, N.K. and E.B. Pasquale, *Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins.* Cell Signal, 2004. **16**(6): p. 655-66.

10. Fang, W.B., et al., *Overexpression of EPHA2 receptor destabilizes adherens junctions via a RhoA-dependent mechanism.* J Cell Sci, 2008. **121**(Pt 3): p. 358-68.

11. Fang, W.B., et al., *A kinase-dependent role for EphA2 receptor in promoting tumor growth and metastasis.* Oncogene, 2005. **24**(53): p. 7859-68.

12. Menges, C.W. and D.J. McCance, *Constitutive activation of the Raf-MAPK pathway causes negative feedback inhibition of Ras-PI3K-AKT and cellular arrest through the EphA2 receptor.* Oncogene, 2008. **27**(20): p. 2934-40.

13. Larsen, A.B., et al., *Activation of the EGFR gene target EphA2 inhibits epidermal growth factor-induced cancer cell motility.* Mol Cancer Res, 2007. **5**(3): p. 283-93.

14. Warner, N., L.E. Wybenga-Groot, and T. Pawson, *Analysis of EphA4 receptor tyrosine kinase substrate specificity using peptide-based arrays.* FEBS J, 2008. **275**(10): p. 2561-73.

15. Luo, H., et al., *EphB6 crosslinking results in costimulation of T cells.* J Clin Invest, 2002. **110**(8): p. 1141-50.

16. Salvucci, O., et al., *EphB2 and EphB4 receptors forward signaling promotes SDF-1-induced endothelial cell chemotaxis and branching remodeling.* Blood, 2006. **108**(9): p. 2914-22.

17. Zhuang, G., et al., *Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy.* Cancer Res, 2010. **70**(1): p. 299-308.

18. Pasquale, E.B., *Eph receptor signalling casts a wide net on cell behaviour.* Nat Rev Mol Cell Biol, 2005. **6**(6): p. 462-75.

19. Sharfe, N., et al., *Ephrin stimulation modulates T cell chemotaxis.* Eur J Immunol, 2002. **32**(12): p. 3745-55.

20. Deroanne, C., et al., *EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway.* J Cell Sci, 2003. **116**(Pt 7): p. 1367-76.

21. Tanaka, M., R. Kamata, and R. Sakai, *EphA2 phosphorylates the cytoplasmic tail of Claudin-4 and mediates paracellular permeability.* J Biol Chem, 2005. **280**(51): p. 42375-82.

22. Miao, H., et al., *Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation.* Nat Cell Biol, 2000. **2**(2): p. 62-9.

23. Zantek, N.D., et al., *E-cadherin regulates the function of the EphA2 receptor tyrosine kinase.* Cell Growth Differ, 1999. **10**(9): p. 629-38.

24. Prevost, N., et al., *Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets.* Proc Natl Acad Sci U S A, 2005. **102**(28): p. 9820-5.

25. Yokote, H., et al., *Trans-activation of EphA4 and FGF receptors mediated by direct interactions between their cytoplasmic domains.* Proc Natl Acad Sci U S A, 2005. **102**(52): p. 18866-71.

26. Gu, C. and S. Park, *The EphA8 receptor regulates integrin activity through p110gamma phosphatidylinositol-3 kinase in a tyrosine kinase activity-independent manner.* Mol Cell Biol, 2001. **21**(14): p. 4579-97.

27. Dalva, M.B., et al., *EphB receptors interact with NMDA receptors and regulate excitatory synapse formation.* Cell, 2000. **103**(6): p. 945-56.

28. Cortina, C., et al., *EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells.* Nat Genet, 2007. **39**(11): p. 1376-83.

29. Ethell, I.M., et al., *EphB/syndecan-2 signaling in dendritic spine morphogenesis.* Neuron, 2001. **31**(6): p. 1001-13.

30. Zisch, A.H., et al., *Tyrosine phosphorylation of L1 family adhesion molecules: implication of the Eph kinase Cek5.* J Neurosci Res, 1997. **47**(6): p. 655-65.

31. Trivier, E. and T.S. Ganesan, *RYK, a catalytically inactive receptor tyrosine kinase, associates with EphB2 and EphB3 but does not interact with AF-6.* J Biol Chem, 2002. **277**(25): p. 23037-43.

32. Egea, J. and R. Klein, *Bidirectional Eph-ephrin signaling during axon guidance.* Trends Cell Biol, 2007. **17**(5): p. 230-8.

33. Davy, A., et al., *Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion.* Genes Dev, 1999. **13**(23): p. 3125-35.

34. Davy, A. and S.M. Robbins, *Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner.* EMBO J, 2000. **19**(20): p. 5396-405.

35. Noren, N.K., et al., *The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl-Crk pathway.* Nat Cell Biol, 2006. **8**(8): p. 815-25.

36. Miao, H., et al., *Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway.* Nat Cell Biol, 2001. **3**(5): p. 527-30.

37. Miao, H., et al., *EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt.* Cancer Cell, 2009. **16**(1): p. 9-20.

38. Shamah, S.M., et al., *EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin.* Cell, 2001. **105**(2): p. 233-44.

39. Fukai, J., et al., *EphA4 promotes cell proliferation and migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line.* Mol Cancer Ther, 2008. **7**(9): p. 2768-78.

40. Adams, R.H. and R. Klein, *Eph receptors and ephrin ligands. essential mediators of vascular development.* Trends Cardiovasc Med, 2000. **10**(5): p. 183-8.

41. Flanagan, J.G. and P. Vanderhaeghen, *The ephrins and Eph receptors in neural development.* Annu Rev Neurosci, 1998. **21**: p. 309-45.

42. Cheng, H.J. and J.G. Flanagan, *Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases.* Cell, 1994. **79**(1): p. 157-68.

43. Drescher, U., et al., *In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases.* Cell, 1995. **82**(3): p. 359-70.

44. Flenniken, A.M., et al., *Distinct and overlapping expression patterns of ligands for Eph-related receptor tyrosine kinases during mouse embryogenesis.* Dev Biol, 1996. **179**(2): p. 382-401.

45. McBride, J.L. and J.C. Ruiz, *Ephrin-A1 is expressed at sites of vascular development in the mouse.* Mech Dev, 1998. **77**(2): p. 201-4.

46. Takahashi, H. and T. Ikeda, *Molecular cloning and expression of rat and mouse B61 gene: implications on organogenesis.* Oncogene, 1995. **11**(5): p. 879-83.

47. Frieden, L.A., et al., *Regulation of heart valve morphogenesis by Eph receptor ligand, ephrin-A1*. Dev Dyn, 2010. **239**(12): p. 3226-34.

48. Wang, H.U., Z.F. Chen, and D.J. Anderson, *Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4.* Cell, 1998. **93**(5): p. 741-53.

49. Adams, R.H., et al., *Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis.* Genes Dev, 1999. **13**(3): p. 295-306.

50. Gerety, S.S., et al., *Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development.* Mol Cell, 1999. **4**(3): p. 403-14.

51. Duffy, S.L., et al., *Expression analysis of the Epha1 receptor tyrosine kinase and its high-affinity ligands Efna1 and Efna3 during early mouse development.* Gene Expr Patterns, 2006. **6**(7): p. 719-23.

52. Munoz, J.J., et al., *Thymic alterations in EphA4-deficient mice.* J Immunol, 2006. **177**(2): p. 804-13.

53. Miao, H., et al., *EphA kinase activation regulates HGF-induced epithelial branching morphogenesis.* J Cell Biol, 2003. **162**(7): p. 1281-92.

54. Miao, H. and B. Wang, *Eph/ephrin signaling in epithelial development and homeostasis.* Int J Biochem Cell Biol, 2009. **41**(4): p. 762-70.

55. Batlle, E., et al., *Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB.* Cell, 2002. **111**(2): p. 251-63.

56. Hens, J.R. and J.J. Wysolmerski, *Key stages of mammary gland development: molecular mechanisms involved in the formation of the embryonic mammary gland.* Breast Cancer Res, 2005. **7**(5): p. 220-4.

57. Sternlicht, M.D., et al., *Hormonal and local control of mammary branching morphogenesis.* Differentiation, 2006. **74**(7): p. 365-81.

58. Sternlicht, M.D., et al., *Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin.* Development, 2005. **132**(17): p. 3923-33.

59. Williams, J.M. and C.W. Daniel, *Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis.* Dev Biol, 1983. **97**(2): p. 274-90.

60. Smith, G.H. and G. Chepko, *Mammary epithelial stem cells.* Microsc Res Tech, 2001. **52**(2): p. 190-203.

61. Kordon, E.C. and G.H. Smith, *An entire functional mammary gland may comprise the progeny from a single cell.* Development, 1998. **125**(10): p. 1921-30.

62. Kim, N.D., et al., *Stem cell characteristics of transplanted rat mammary clonogens.* Exp Cell Res, 2000. **260**(1): p. 146-59.

63. DeOme, K.B. and D. Medina, *A new approach to mammary tumorigenesis in rodents.* Cancer, 1969. **24**(6): p. 1255-8.

64. Hennighausen, L. and G.W. Robinson, *Information networks in the mammary gland.* Nat Rev Mol Cell Biol, 2005. **6**(9): p. 715-25.

65. Nikolova, Z., et al., *Cell-type specific and estrogen dependent expression of the receptor tyrosine kinase EphB4 and its ligand ephrin-B2 during mammary gland morphogenesis.* J Cell Sci, 1998. **111 (Pt 18)**: p. 2741-51.

66. Munarini, N., et al., *Altered mammary epithelial development, pattern formation and involution in transgenic mice expressing the EphB4 receptor tyrosine kinase.* J Cell Sci, 2002. **115**(Pt 1): p. 25-37.

67. Kouros-Mehr, H. and Z. Werb, *Candidate regulators of mammary branching morphogenesis identified by genome-wide transcript analysis.* Dev Dyn, 2006. **235**(12): p. 3404-12.

68. Andres, A.C., et al., *Expression of two novel eph-related receptor protein tyrosine kinases in mammary gland development and carcinogenesis.* Oncogene, 1994. **9**(5): p. 1461-7.

69. Vaught, D., J. Chen, and D.M. Brantley-Sieders, *Regulation of mammary gland branching morphogenesis by EphA2 receptor tyrosine kinase.* Mol Biol Cell, 2009. **20**(10): p. 2572-81.

70. Ogawa, K., et al., *The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization.* Oncogene, 2000. **19**(52): p. 6043-52.

71. Zelinski, D.P., et al., *EphA2 overexpression causes tumorigenesis of mammary epithelial cells.* Cancer Res, 2001. **61**(5): p. 2301-6.

72. Martin, K.J., et al., *Prognostic breast cancer signature identified from 3D culture model accurately predicts clinical outcome across independent datasets.* PLoS One, 2008. **3**(8): p. e2994.

73. Fournier, M.V., et al., *Gene expression signature in organized and growth-arrested mammary acini predicts good outcome in breast cancer.* Cancer Res, 2006. **66**(14): p. 7095-102.

74. Ireton, R.C. and J. Chen, *EphA2 receptor tyrosine kinase as a promising target for cancer therapeutics.* Curr Cancer Drug Targets, 2005. **5**(3): p. 149-57.

75. Brantley-Sieders, D., et al., *Eph receptor tyrosine kinases in tumor and tumor microenvironment.* Curr Pharm Des, 2004. **10**(27): p. 3431-42.

76. Brannan, J.M., et al., *Expression of the receptor tyrosine kinase EphA2 is increased in smokers and predicts poor survival in non-small cell lung cancer.* Clin Cancer Res, 2009. **15**(13): p. 4423-30.

77. Miyazaki, T., et al., *EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma.* Int J Cancer, 2003. **103**(5): p. 657-63.

78. Wu, D., et al., *Prognostic value of EphA2 and EphrinA-1 in squamous cell cervical carcinoma.* Gynecol Oncol, 2004. **94**(2): p. 312-9.

79. Herrem, C.J., et al., *Expression of EphA2 is prognostic of disease-free interval and overall survival in surgically treated patients with renal cell carcinoma.* Clin Cancer Res, 2005. **11**(1): p. 226-31.

80. Wang, L.F., et al., *Increased expression of EphA2 correlates with adverse outcome in primary and recurrent glioblastoma multiforme patients.* Oncol Rep, 2008. **19**(1): p. 151-6.

81. Kamat, A.A., et al., *EphA2 overexpression is associated with lack of hormone receptor expression and poor outcome in endometrial cancer.* Cancer, 2009. **115**(12): p. 2684-92.

82. Oki, M., et al., *Overexpression of the receptor tyrosine kinase EphA4 in human gastric cancers.* World J Gastroenterol, 2008. **14**(37): p. 5650-6.

83. Wang, L.F., et al., *Increased expression of EphA7 correlates with adverse outcome in primary and recurrent glioblastoma multiforme patients.* BMC Cancer, 2008. **8**: p. 79.

84. Kumar, S.R., et al., *The receptor tyrosine kinase EphB4 is overexpressed in ovarian cancer, provides survival signals and predicts poor outcome.* Br J Cancer, 2007. **96**(7): p. 1083-91.

85. Masood, R., et al., *EphB4 provides survival advantage to squamous cell carcinoma of the head and neck.* Int J Cancer, 2006. **119**(6): p. 1236-48.

86. Fox, B.P. and R.P. Kandpal, *Invasiveness of breast carcinoma cells and transcript profile: Eph receptors and ephrin ligands as molecular markers of potential diagnostic and prognostic application.* Biochem Biophys Res Commun, 2004. **318**(4): p. 882-92.

87. Wu, Q., et al., *Expression of Ephb2 and Ephb4 in breast carcinoma.* Pathol Oncol Res, 2004. **10**(1): p. 26-33.

88. Brantley, D.M., et al., *Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo.* Oncogene, 2002. **21**(46): p. 7011-26.

89. Duxbury, M.S., et al., *EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma.* Oncogene, 2004. **23**(7): p. 1448-56.

90. Landen, C.N., Jr., et al., *Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery.* Cancer Res, 2005. **65**(15): p. 6910-8.

91. Nasreen, N., K.A. Mohammed, and V.B. Antony, *Silencing the receptor EphA2 suppresses the growth and haptotaxis of malignant mesothelioma cells.* Cancer, 2006. **107**(10): p. 2425-35.

92. Kumar, S.R., et al., *Receptor tyrosine kinase EphB4 is a survival factor in breast cancer.* Am J Pathol, 2006. **169**(1): p. 279-93.

93. van de Vijver, M.J., et al., *A gene-expression signature as a predictor of survival in breast cancer.* N Engl J Med, 2002. **347**(25): p. 1999-2009.

94. Kikawa, K.D., et al., *Regulation of the EphA2 kinase by the low molecular weight tyrosine phosphatase induces transformation.* J Biol Chem, 2002. **277**(42): p. 39274-9.

95. Orsulic, S. and R. Kemler, *Expression of Eph receptors and ephrins is differentially regulated by E-cadherin.* J Cell Sci, 2000. **113 (Pt 10)**: p. 1793-802.

96. Marquardt, T., et al., *Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains.* Cell, 2005. **121**(1): p. 127-39.

97. Noren, N.K. and E.B. Pasquale, *Paradoxes of the EphB4 receptor in cancer.* Cancer Res, 2007. **67**(9): p. 3994-7.

98. Duxbury, M.S., et al., *Ligation of EphA2 by Ephrin A1-Fc inhibits pancreatic adenocarcinoma cellular invasiveness.* Biochem Biophys Res Commun, 2004. **320**(4): p. 1096-102.

99. Hu, M., et al., *EphA2 induction of fibronectin creates a permissive microenvironment for malignant cells.* Mol Cancer Res, 2004. **2**(10): p. 533-40.

100. Guo, H., et al., *Disruption of EphA2 receptor tyrosine kinase leads to increased susceptibility to carcinogenesis in mouse skin.* Cancer Res, 2006. **66**(14): p. 7050-8.

101. Batlle, E., et al., *EphB receptor activity suppresses colorectal cancer progression.* Nature, 2005. **435**(7045): p. 1126-30.

102. Folkman, J., *Role of angiogenesis in tumor growth and metastasis.* Semin Oncol, 2002. **29**(6 Suppl 16): p. 15-8.

103. Pandey, A., et al., *Role of B61, the ligand for the Eck receptor tyrosine kinase, in TNF-alpha-induced angiogenesis.* Science, 1995. **268**(5210): p. 567-9.

104. Casanovas, O., et al., *Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors.* Cancer Cell, 2005. **8**(4): p. 299-309.

105. Brantley-Sieders, D.M., et al., *Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression.* FASEB J, 2005. **19**(13): p. 1884-6.

106. Brantley-Sieders, D.M., et al., *EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation.* J Cell Sci, 2004. **117**(Pt 10): p. 2037-49.

107. Hunter, S.G., et al., *Essential role of Vav family guanine nucleotide exchange factors in EphA receptor-mediated angiogenesis.* Mol Cell Biol, 2006. **26**(13): p. 4830-42.

108. Shin, D., et al., *Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization.* Dev Biol, 2001. **230**(2): p. 139-50.

109. Noren, N.K., et al., *Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5583-8.

110. Martiny-Baron, G., et al., *Inhibition of tumor growth and angiogenesis by soluble EphB4.* Neoplasia, 2004. **6**(3): p. 248-57.

111. Sawai, Y., et al., *Expression of ephrin-B1 in hepatocellular carcinoma: possible involvement in neovascularization.* J Hepatol, 2003. **39**(6): p. 991-6.

112. Nagashima, T., et al., *Phosphoproteome and transcriptome analyses of ErbB ligand-stimulated MCF-7 cells.* Cancer Genomics Proteomics, 2008. **5**(3-4): p. 161-8.

113. Carles-Kinch, K., et al., *Antibody targeting of the EphA2 tyrosine kinase inhibits malignant cell behavior.* Cancer Res, 2002. **62**(10): p. 2840-7.

114. Mundy, G.R., *Metastasis to bone: causes, consequences and therapeutic opportunities.* Nat Rev Cancer, 2002. **2**(8): p. 584-93.

115. Chambers, A.F., A.C. Groom, and I.C. MacDonald, *Dissemination and growth of cancer cells in metastatic sites.* Nat Rev Cancer, 2002. **2**(8): p. 563-72.

116. Massague, J., *Sorting out breast-cancer gene signatures.* N Engl J Med, 2007. **356**(3): p. 294-7.

117. Paget, S., *The distribution of secondary growths in cancer of the breast.* Lancet, 1889. **1**: p. 571-573.

118. Mundy, G.R., *Mechanisms of bone metastasis.* Cancer, 1997. **80**(8 Suppl): p. 1546-56.

119. Hadjidakis, D.J. and Androulakis, II, *Bone remodeling.* Ann N Y Acad Sci, 2006. **1092**: p. 385-96.

120. Karsenty, G., *The complexities of skeletal biology.* Nature, 2003. **423**(6937): p. 316-8.

121. Ara, T. and Y.A. DeClerck, *Mechanisms of invasion and metastasis in human neuroblastoma.* Cancer Metastasis Rev, 2006. **25**(4): p. 645-57.

122. Karsenty, G. and E.F. Wagner, *Reaching a genetic and molecular understanding of skeletal development.* Dev Cell, 2002. **2**(4): p. 389-406.

123. Bussard, K.M., C.V. Gay, and A.M. Mastro, *The bone microenvironment in metastasis; what is special about bone?* Cancer Metastasis Rev, 2008. **27**(1): p. 41-55.

124. Bergfeld, S.A. and Y.A. DeClerck, *Bone marrow-derived mesenchymal stem cells and the tumor microenvironment.* Cancer Metastasis Rev, 2010. **29**(2): p. 249-61.

125. Tatsumi, S., et al., *Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction.* Cell Metab, 2007. **5**(6): p. 464-75.

126. Bonewald, L.F., *Osteocytes as dynamic multifunctional cells.* Ann N Y Acad Sci, 2007. **1116**: p. 281-90.

127. Teitelbaum, S.L., *Osteoclasts; culprits in inflammatory osteolysis.* Arthritis Res Ther, 2006. **8**(1): p. 201.

128. Teitelbaum, S.L., *Osteoclasts: what do they do and how do they do it?* Am J Pathol, 2007. **170**(2): p. 427-35.

129. Kodama, H., et al., *Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells.* J Exp Med, 1991. **173**(5): p. 1291-4.

130. Teitelbaum, S.L. and F.P. Ross, *Genetic regulation of osteoclast development and function.* Nat Rev Genet, 2003. **4**(8): p. 638-49.

131. Zaidi, M., *Skeletal remodeling in health and disease.* Nat Med, 2007. **13**(7): p. 791-801.

132. Matsuo, K. and N. Irie, *Osteoclast-osteoblast communication.* Arch Biochem Biophys, 2008. **473**(2): p. 201-9.

133. Pfeilschifter, J. and G.R. Mundy, *Modulation of type beta transforming growth factor activity in bone cultures by osteotropic hormones.* Proc Natl Acad Sci U S A, 1987. **84**(7): p. 2024-8.

134. Kong, Y.Y., et al., *OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis.* Nature, 1999. **397**(6717): p. 315-23.

135. Li, J., et al., *RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism.* Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1566-71.

136. Simonet, W.S., et al., *Osteoprotegerin: a novel secreted protein involved in the regulation of bone density.* Cell, 1997. **89**(2): p. 309-19.

137. Guise, T.A. and G.R. Mundy, *Cancer and bone.* Endocr Rev, 1998. **19**(1): p. 18-54.

138. Compagni, A., et al., *Control of skeletal patterning by ephrinB1-EphB interactions.* Dev Cell, 2003. **5**(2): p. 217-30.

139. Davy, A., J. Aubin, and P. Soriano, *Ephrin-B1 forward and reverse signaling are required during mouse development.* Genes Dev, 2004. **18**(5): p. 572-83.

140. Xing, W., et al., *Ephrin B1 regulates bone marrow stromal cell differentiation and bone formation by influencing TAZ transactivation via complex formation with NHERF1.* Mol Cell Biol, 2010. **30**(3): p. 711-21.

141. Wieland, I., et al., *Mutations of the ephrin-B1 gene cause craniofrontonasal syndrome.* Am J Hum Genet, 2004. **74**(6): p. 1209-15.

142. Twigg, S.R., et al., *Mutations of ephrin-B1 (EFNB1), a marker of tissue boundary formation, cause craniofrontonasal syndrome.* Proc Natl Acad Sci U S A, 2004. **101**(23): p. 8652-7.

143. Zhao, C., et al., *Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis.* Cell Metab, 2006. **4**(2): p. 111-21.

144. Ting, M.C., et al., *EphA4 as an effector of Twist1 in the guidance of osteogenic precursor cells during calvarial bone growth and in craniosynostosis.* Development, 2009. **136**(5): p. 855-64.

145. Irie, N., et al., *Bidirectional signaling through ephrinA2-EphA2 enhances osteoclastogenesis and suppresses osteoblastogenesis.* J Biol Chem, 2009. **284**(21): p. 14637-44.

146. Liao, J. and L.K. McCauley, *Skeletal metastasis: Established and emerging roles of parathyroid hormone related protein (PTHrP).* Cancer Metastasis Rev, 2006. **25**(4): p. 559-71.

147. Morrissey, C., et al., *Differential expression of angiogenesis associated genes in prostate cancer bone, liver and lymph node metastases.* Clin Exp Metastasis, 2008. **25**(4): p. 377-88.

148. Guenther, R., et al., *Giant cell tumors of the bone: molecular profiling and expression analysis of Ephrin A1 receptor, Claudin 7, CD52, FGFR3 and AMFR.* Pathol Res Pract, 2005. **201**(10): p. 649-63.

149. Taddei, M.L., et al., *Kinase-dependent and -independent roles of EphA2 in the regulation of prostate cancer invasion and metastasis.* Am J Pathol, 2009. **174**(4): p. 1492-503.

150. Sims, N.A. and J.H. Gooi, *Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption.* Semin Cell Dev Biol, 2008. **19**(5): p. 444-51.

151. Guise, T.A. and J.M. Chirgwin, *Transforming growth factor-beta in osteolytic breast cancer bone metastases.* Clin Orthop Relat Res, 2003(415 Suppl): p. S32-8.

152. Pederson, L., et al., *Identification of breast cancer cell line-derived paracrine factors that stimulate osteoclast activity.* Cancer Res, 1999. **59**(22): p. 5849-55.

153. Jimi, E., et al., *Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells.* Exp Cell Res, 1999. **247**(1): p. 84-93.

154. Coffman, K.T., et al., *Differential EphA2 epitope display on normal versus malignant cells.* Cancer Res, 2003. **63**(22): p. 7907-12.

155. Hammond, S.A., et al., *Selective targeting and potent control of tumor growth using an EphA2/CD3-Bispecific single-chain antibody construct.* Cancer Res, 2007. **67**(8): p. 3927-35.

156. Wykosky, J., D.M. Gibo, and W. Debinski, *A novel, potent, and specific ephrinA1-based cytotoxin against EphA2 receptor expressing tumor cells.* Mol Cancer Ther, 2007. **6**(12 Pt 1): p. 3208-18.

157. Jackson, D., et al., *A human antibody-drug conjugate targeting EphA2 inhibits tumor growth in vivo.* Cancer Res, 2008. **68**(22): p. 9367-74.

158. Landen, C.N., et al., *Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer.* Cancer Biol Ther, 2006. **5**(12): p. 1708-13.

159. Cheng, N., et al., *Inhibition of VEGF-dependent multistage carcinogenesis by soluble EphA receptors.* Neoplasia, 2003. **5**(5): p. 445-56.

160. Dobrzanski, P., et al., *Antiangiogenic and antitumor efficacy of EphA2 receptor antagonist.* Cancer Res, 2004. **64**(3): p. 910-9.

161. Noberini, R., et al., *Small molecules can selectively inhibit ephrin binding to the EphA4 and EphA2 receptors.* J Biol Chem, 2008. **283**(43): p. 29461-72.

162. Chrencik, J.E., et al., *Three-dimensional structure of the EphB2 receptor in complex with an antagonistic peptide reveals a novel mode of inhibition.* J Biol Chem, 2007. **282**(50): p. 36505-13.

163. Tatsumi, T., et al., *Disease stage variation in CD4+ and CD8+ T-cell reactivity to the receptor tyrosine kinase EphA2 in patients with renal cell carcinoma.* Cancer Res, 2003. **63**(15): p. 4481-9.

164. Yamaguchi, S., et al., *Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide-pulsed dendritic cell vaccines.* Cancer, 2007. **110**(7): p. 1469-77.

165. Hatano, M., et al., *Vaccination with EphA2-derived T cell-epitopes promotes immunity against both EphA2-expressing and EphA2-negative tumors.* J Transl Med, 2004. **2**(1): p. 40.

166. Pasquale, E.B., *Eph receptors and ephrins in cancer: bidirectional signalling and beyond.* Nat Rev Cancer, 2010. **10**(3): p. 165-80.

167. Vaught, D., D.M. Brantley-Sieders, and J. Chen, *Eph receptors in breast cancer: roles in tumor promotion and tumor suppression.* Breast Cancer Res, 2008. **10**(6): p. 217.

168. Bardelle, C., et al., *Inhibitors of the tyrosine kinase EphB4. Part 1: Structure-based design and optimization of a series of 2,4-bis-anilinopyrimidines.* Bioorg Med Chem Lett, 2008. **18**(9): p. 2776-80.

169. Miyazaki, Y., et al., *Design and effective synthesis of novel templates, 3,7-diphenyl-4-amino-thieno and furo-[3,2-c]pyridines as protein kinase inhibitors and in vitro evaluation targeting angiogenetic kinases.* Bioorg Med Chem Lett, 2007. **17**(1): p. 250-4.

170. Bardelle, C., et al., *Inhibitors of the tyrosine kinase EphB4. Part 2: structure-based discovery and optimisation of 3,5-bis substituted anilinopyrimidines.* Bioorg Med Chem Lett, 2008. **18**(21): p. 5717-21.

171. Lafleur, K., et al., *Structure-based optimization of potent and selective inhibitors of the tyrosine kinase erythropoietin producing human hepatocellular carcinoma receptor B4 (EphB4).* J Med Chem, 2009. **52**(20): p. 6433-46.

172. Kolb, P., et al., *Discovery of kinase inhibitors by high-throughput docking and scoring based on a transferable linear interaction energy model.* J Med Chem, 2008. **51**(5): p. 1179-88.

173. Wang, X.D., et al., *Identification of candidate predictive and surrogate molecular markers for dasatinib in prostate cancer: rationale for patient selection and efficacy monitoring.* Genome Biol, 2007. **8**(11): p. R255.

174. Huang, F., et al., *Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection.* Cancer Res, 2007. **67**(5): p. 2226-38.

175. Karaman, M.W., et al., *A quantitative analysis of kinase inhibitor selectivity.* Nat Biotechnol, 2008. **26**(1): p. 127-32.

176. Choi, Y., et al., *Discovery and structural analysis of Eph receptor tyrosine kinase inhibitors.* Bioorg Med Chem Lett, 2009. **19**(15): p. 4467-70.

177. Shahzad, M.M., et al., *Dual targeting of EphA2 and FAK in ovarian carcinoma.* Cancer Biol Ther, 2009. **8**(11): p. 1027-34.

178. Xia, G., et al., *EphB4 receptor tyrosine kinase is expressed in bladder cancer and provides signals for cell survival.* Oncogene, 2006. **25**(5): p. 769-80.

179. Xia, G., et al., *EphB4 expression and biological significance in prostate cancer.* Cancer Res, 2005. **65**(11): p. 4623-32.

180. Kumar, S.R., et al., *Preferential induction of EphB4 over EphB2 and its implication in colorectal cancer progression.* Cancer Res, 2009. **69**(9): p. 3736-45.

181. Cheng, N., et al., *Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis.* Mol Cancer Res, 2002. **1**(1): p. 2-11.

182. Kertesz, N., et al., *The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth.* Blood, 2006. **107**(6): p. 2330-8.

183. Scehnet, J.S., et al., *The role of Ephs, Ephrins, and growth factors in Kaposi sarcoma and implications of EphrinB2 blockade.* Blood, 2009. **113**(1): p. 254-63.

184. Murai, K.K., et al., *Targeting the EphA4 receptor in the nervous system with biologically active peptides.* Mol Cell Neurosci, 2003. **24**(4): p. 1000-11.

185. Koolpe, M., et al., *EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity.* J Biol Chem, 2005. **280**(17): p. 17301-11.

186. Salvucci, O., et al., *EphrinB reverse signaling contributes to endothelial and mural cell assembly into vascular structures.* Blood, 2009. **114**(8): p. 1707-16.

187. Chrencik, J.E., et al., *Structure and thermodynamic characterization of the EphB4/Ephrin-B2 antagonist peptide complex reveals the determinants for receptor specificity.* Structure, 2006. **14**(2): p. 321-30.

188. Qin, H., et al., *Crystal structure and NMR binding reveal that two small molecule antagonists target the high affinity ephrin-binding channel of the EphA4 receptor.* J Biol Chem, 2008. **283**(43): p. 29473-84.

189. Mao, W., et al., *EphB2 as a therapeutic antibody drug target for the treatment of colorectal cancer.* Cancer Res, 2004. **64**(3): p. 781-8.

190. Landen, C.N., Jr., et al., *Efficacy and antivascular effects of EphA2 reduction with an agonistic antibody in ovarian cancer.* J Natl Cancer Inst, 2006. **98**(21): p. 1558-70.

191. Bruckheimer, E.M., et al., *Antibody-dependent cell-mediated cytotoxicity effector-enhanced EphA2 agonist monoclonal antibody demonstrates potent activity against human tumors.* Neoplasia, 2009. **11**(6): p. 509-17, 2 p following 517.

192. Kiewlich, D., et al., *Anti-EphA2 antibodies decrease EphA2 protein levels in murine CT26 colorectal and human MDA-231 breast tumors but do not inhibit tumor growth.* Neoplasia, 2006. **8**(1): p. 18-30.

193. van Geer, M.A., et al., *Ephrin A2 receptor targeting does not increase adenoviral pancreatic cancer transduction in vivo.* World J Gastroenterol, 2009. **15**(22): p. 2754-62.

194. Noblitt, L.W., et al., *Decreased tumorigenic potential of EphA2-overexpressing breast cancer cells following treatment with adenoviral vectors that express EphrinA1.* Cancer Gene Ther, 2004. **11**(11): p. 757-66.

195. Gobin, A.M., J.J. Moon, and J.L. West, *EphrinA I-targeted nanoshells for photothermal ablation of prostate cancer cells.* Int J Nanomedicine, 2008. **3**(3): p. 351-8.

196. Cai, W., et al., *Quantitative radioimmunoPET imaging of EphA2 in tumor-bearing mice.* Eur J Nucl Med Mol Imaging, 2007. **34**(12): p. 2024-36.

197. Scarberry, K.E., et al., *Selective removal of ovarian cancer cells from human ascites fluid using magnetic nanoparticles.* Nanomedicine, 2009. **6**(3): p. 399-408.

198. Scarberry, K.E., et al., *Magnetic nanoparticle-peptide conjugates for in vitro and in vivo targeting and extraction of cancer cells.* J Am Chem Soc, 2008. **130**(31): p. 10258-62.

199. Vearing, C., et al., *Concurrent binding of anti-EphA3 antibody and ephrin-A5 amplifies EphA3 signaling and downstream responses: potential as EphA3-specific tumor-targeting reagents.* Cancer Res, 2005. **65**(15): p. 6745-54.

200. Scott, J.D. and T. Pawson, *Cell signaling in space and time: where proteins come together and when they're apart.* Science, 2009. **326**(5957): p. 1220-4.

201. Watson, C.J. and W.T. Khaled, *Mammary development in the embryo and adult: a journey of morphogenesis and commitment.* Development, 2008. **135**(6): p. 995-1003.

202. Pollard, J.W., *Tumour-stromal interactions. Transforming growth factor-beta isoforms and hepatocyte growth factor/scatter factor in mammary gland ductal morphogenesis.* Breast Cancer Res, 2001. **3**(4): p. 230-7.

203. Kamalati, T., et al., *HGF/SF in mammary epithelial growth and morphogenesis: in vitro and in vivo models.* J Mammary Gland Biol Neoplasia, 1999. **4**(1): p. 69-77.

204. Andres, A.C., et al., *EphB4 receptor tyrosine kinase transgenic mice develop glomerulopathies reminiscent of aglomerular vascular shunts.* Mech Dev, 2003. **120**(4): p. 511-6.

205. Murai, K.K., et al., *Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling.* Nat Neurosci, 2003. **6**(2): p. 153-60.

206. Brantley-Sieders, D.M. and J. Chen, *Eph receptor tyrosine kinases in angiogenesis: from development to disease.* Angiogenesis, 2004. **7**(1): p. 17-28.

207. Poliakov, A., M. Cotrina, and D.G. Wilkinson, *Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly.* Dev Cell, 2004. **7**(4): p. 465-80.

208. Kinch, M.S. and K. Carles-Kinch, *Overexpression and functional alterations of the EphA2 tyrosine kinase in cancer.* Clin Exp Metastasis, 2003. **20**(1): p. 59-68.

209. Brantley, D.M., et al., *Nuclear factor-kappaB (NF-kappaB) regulates proliferation and branching in mouse mammary epithelium.* Mol Biol Cell, 2001. **12**(5): p. 1445-55.

210. Debnath, J., S.J. Walker, and J.S. Brugge, *Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner.* J Cell Biol, 2003. **163**(2): p. 315-26.

211. Muraoka, R.S., et al., *Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1.* Mol Cell Biol, 2003. **23**(23): p. 8691-703.

212. Wiseman, B.S. and Z. Werb, *Stromal effects on mammary gland development and breast cancer.* Science, 2002. **296**(5570): p. 1046-9.

213. Ridley, A.J., *Rho family proteins: coordinating cell responses.* Trends Cell Biol, 2001. **11**(12): p. 471-7.

214. Ridley, A.J., *Rho GTPases and cell migration.* J Cell Sci, 2001. **114**(Pt 15): p. 2713-22.

215. Ridley, A.J., *Rho proteins, PI 3-kinases, and monocyte/macrophage motility.* FEBS Lett, 2001. **498**(2-3): p. 168-71.

216. Ewald, A.J., et al., *Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis.* Dev Cell, 2008. **14**(4): p. 570-81.

217. Sternlicht, M.D., *Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis.* Breast Cancer Res, 2006. **8**(1): p. 201.

218. Chakravarty, G., et al., *P190-B, a Rho-GTPase-activating protein, is differentially expressed in terminal end buds and breast cancer.* Cell Growth Differ, 2000. **11**(7): p. 343-54.

219. Vargo-Gogola, T., et al., *P190-B Rho GTPase-activating protein overexpression disrupts ductal morphogenesis and induces hyperplastic lesions in the developing mammary gland.* Mol Endocrinol, 2006. **20**(6): p. 1391-405.

220. Coleman, R.E., et al., *Biochemical prediction of response of bone metastases to treatment.* Br J Cancer, 1988. **58**(2): p. 205-10.

221. Davy, A. and P. Soriano, *Ephrin signaling in vivo: look both ways.* Dev Dyn, 2005. **232**(1): p. 1-10.

222. Lynch, C.C., et al., *MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL.* Cancer Cell, 2005. **7**(5): p. 485-96.

223. Thiolloy, S., et al., *Osteoclast-derived matrix metalloproteinase-7, but not matrix metalloproteinase-9, contributes to tumor-induced osteolysis.* Cancer Res, 2009. **69**(16): p. 6747-55.

224. Mitsiades, C.S., et al., *The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple myeloma: interplay of growth factors, their receptors and stromal interactions.* Eur J Cancer, 2006. **42**(11): p. 1564-73.

225. Fukaya, Y., et al., *Identification of galectin-3-binding protein as a factor secreted by tumor cells that stimulates interleukin-6 expression in the bone marrow stroma.* J Biol Chem, 2008. **283**(27): p. 18573-81.

226. Chen, J., et al., *Eph receptors and Ephrins in cancer: common themes and controversies.* Cancer Res, 2008. **68**(24): p. 10031-3.

227. Pasquale, E.B., *Eph-ephrin promiscuity is now crystal clear.* Nat Neurosci, 2004. **7**(5): p. 417-8.

228. Lee, H.S., et al., *EphrinB1 controls cell-cell junctions through the Par polarity complex.* Nat Cell Biol, 2008. **10**(8): p. 979-86.

229. Li, J.J., et al., *EphrinA5 acts as a tumor suppressor in glioma by negative regulation of epidermal growth factor receptor.* Oncogene, 2009. **28**(15): p. 1759-68.

230. Campbell, T.N., et al., *Ephrin A5 expression promotes invasion and transformation of murine fibroblasts.* Biochem Biophys Res Commun, 2006. **350**(3): p. 623-8.

231. Xu, N.J. and M. Henkemeyer, *Ephrin-B3 reverse signaling through Grb4 and cytoskeletal regulators mediates axon pruning.* Nat Neurosci, 2009. **12**(3): p. 268-76.

232. Jiang, G., et al., *In human leukemia cells ephrin-B-induced invasive activity is supported by Lck and is associated with reassembling of lipid raft signaling complexes.* Mol Cancer Res, 2008. **6**(2): p. 291-305.

233. Nakada, M., et al., *Ephrin-B3 ligand promotes glioma invasion through activation of Rac1.* Cancer Res, 2006. **66**(17): p. 8492-500.

234. Lindberg, R.A. and T. Hunter, *cDNA cloning and characterization of eck, an epithelial cell receptor protein-tyrosine kinase in the eph/elk family of protein kinases.* Mol Cell Biol, 1990. **10**(12): p. 6316-24.

235. Cheng, N., D.M. Brantley, and J. Chen, *The ephrins and Eph receptors in angiogenesis.* Cytokine Growth Factor Rev, 2002. **13**(1): p. 75-85.

236. Garcia, T., et al., *A convenient clinically relevant model of human breast cancer bone metastasis.* Clin Exp Metastasis, 2008. **25**(1): p. 33-42.

237. Kang, Y., et al., *A multigenic program mediating breast cancer metastasis to bone.* Cancer Cell, 2003. **3**(6): p. 537-49.

238. Eckhardt, B.L., et al., *Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix.* Mol Cancer Res, 2005. **3**(1): p. 1-13.